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(54) Title: BIOLOGICALLY ACTIVE GLYCOPROTEIN HORMONES PRODUCED IN PROCARYOTIC CELLS			
(57) Abstract This invention provides biologically active recombinant glycoprotein hormones produced in procaryotic cells, and methods to produce such hormones. Unfolded glycoprotein hormone subunits are expressed in procaryotic cells, then re-folded <i>in vitro</i> in a thiol redox buffer to form assembly-competent subunits. The subunits are assembled to produce active hormones, which, depending on the nature of their glycosylation, may be used as substitutes for native glycoprotein hormones or as glycoprotein hormone antagonists.			

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**BIOLOGICALLY ACTIVE GLYCOPROTEIN HORMONES
PRODUCED IN PROCARYOTIC CELLS**

Pursuant to 35 U.S.C. §202(c), it is hereby
acknowledged that the U.S. Government has certain
rights in the invention described herein, which was
made in part with funds from the National Institutes
5 of Health.

FIELD OF THE INVENTION

This invention relates to the family of
mammalian hormones known as "glycoprotein hormones".
In particular, this invention provides biologically
10 active recombinant glycoprotein hormones produced in
procaryotic cells. The invention further provides
methods for expressing subunits of the hormones in
procaryotic cells and re-folding the subunits in
vitro, to produce biologically active glycoprotein
15 hormones in quantities sufficient for clinical use.

BACKGROUND OF THE INVENTION

The family of mammalian hormones commonly
referred to as the "glycoprotein hormones" includes
the pituitary gonadotropins, luteinizing hormone (LH)
20 and follicle-stimulating hormone (FSH) and the
placental gonadotropin, chorionic gonadotropin (CG),
as well as pituitary thyroid-stimulating hormone
(TSH). The gonadotropins play key roles in various
testicular and ovarian functions by regulating
25 gametogenesis and synthesis of steroid hormones in the
ovaries and testes. Thyroid stimulating hormone
regulates numerous cellular processes in the thyroid
involved in synthesis and secretion of thyroid
hormones, including thyroxine. For a review of the
30 psychological functions of the glycoprotein hormones,
see, generally, Chapter 36 and Chapter 39
(specifically pages 522-523 and 577) in Basic and

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Clinical Pharmacology, 5th edition, B.G. Katzung, ed.,
Appleton & Lange (1992).

Although diverse in function, the glycoprotein hormones share numerous structural similarities. Each glycoprotein hormone is composed of two subunits, referred to as α and β . Within a species, the α subunit is essentially identical among the different hormones of the family, whereas the β subunit is unique to each hormone. Because of its commonality within the glycoprotein hormone family, the α subunit shows a very high degree of amino acid sequence homology from one species to another. Likewise, although β subunits are unique for each specific hormone, β subunits show strong inter-hormone and inter-species sequence homology, particularly in regions involved in protein folding, glycosylation and assembly of $\alpha\beta$ heterodimers. Significant (i.e., approximately ~80%) sequence homology has been found between LH- β and hCG- β . There is also a lesser, but still significant, homology with FSH- β and TSH- β . Comparative amino acid sequences of glycoprotein hormone α and β subunits from a variety of species are set forth in a review by Ryan et al., Recent Progress in Hormone Research, 43: 383-429 (1987).

Both subunits of glycoprotein hormones are glycosylated. The β subunit contains two N-linked oligosaccharides and, in the case of the β subunit of human chorionic gonadotropin (hCG- β), there are four O-linked oligosaccharides in the carboxyl extension of the polypeptide. Apparently, the O-linked chains are not required for biological activity, since they are not present on LH- β subunits. The α subunit contains two N-linked oligosaccharides.

The glycoprotein hormones possess a high degree of disulfide bridging in the α and β subunits. The α subunit contains five disulfide bonds, while

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each of the β subunits in the family contains six disulfide bonds. All the β subunits have identical relative positions of the 12 cysteines that form the six disulfide bonds in the subunit. Detailed
5 descriptions of the polypeptide and carbohydrate structures of the glycoprotein hormones can be found in the following reviews: Ryan et al., 1987, supra.; Ryan et al., FASEB J., 2: 2661-2669 (1988); and Pierce & Parsons, Ann. Rev. Biochem., 50: 465-495 (1981).

10 Glycoprotein hormone α and β subunits assemble to form functional heterodimers through strong, non-covalent interactions. Inter-hormone and inter-species $\alpha\beta$ hybrids have been prepared. It was learned from such preparations that hormonal
15 specificity is dictated substantially by the β subunit; however, both the α and the β subunit are apparently involved to some degree in receptor recognition and binding affinity (i.e., potency).

The glycoprotein hormones elicit their
20 respective biological responses by binding to a plasma membrane receptor on target cells and activating the adenylate cyclase enzyme system by stimulating synthesis of cAMP. Evidence also exists that glycoprotein hormones activate the inositol
25 triphosphate-diacylglycerol system (see Davis et al., Biochem. J., 238: 597-604, 1976). These system activation activities of the glycoprotein hormones are sometimes referred to herein collectively as "signal transduction" activity, a term that also includes the
30 in vivo effects of the hormones, such as stimulation of ovulation.

The carbohydrate moieties on the glycoprotein hormones function to stabilize the hormones in the blood, and also appear to play a role
35 in receptor binding and signal transduction. Experiments with chemically deglycosylated hormones

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indicate that removal of N-linked oligosaccharides does not interfere with receptor binding, but may inhibit signal transduction activity of the hormone. It has been found that removal of N-linked
5 carbohydrate from the α subunit can enhance binding, when the deglycosylated α subunit is combined with a glycosylated β subunit. Keutmann et al.,
Biochemistry, 22: 3067-3072 (1983). Recent studies
10 with N-deglycosylated hCG (using site-directed mutagenesis) have shown a high receptor binding activity for the deglycosylated hormone, but little or no signal transduction ability (i.e., the
deglycosylated hormone is an antagonist of native hormones). Dunkel et al., Endocrinology, 132: 763-769
15 (1993).

The gonadotropin glycoprotein hormones are used clinically to induce ovulation in women and spermatogenesis in men, and for treatment of hypogonadism in both males and females. The pituitary
20 glycoprotein hormones are used clinically for treatment of various pathological conditions associated with underproduction of thyroid hormones. See, e.g., Basic and Clinical Pharmacology, *supra*. Additionally, considerable speculation has developed
25 recently with regard to the use of antagonistic derivatives of glycoprotein hormones for use as anti-fertility agents, and for treatment of diseases and pathological conditions associated with overproduction of steroid hormones (e.g., prolonged or excessive
30 estrogen exposure as a risk factor for endometrial and breast cancer) or thyroid hormones (e.g., hyperthyroidism, Grave's disease). As described above, N- deglycosylated $\alpha\beta$ dimers of hCG have been
shown to be potent antagonists of native hCG.

35 Although glycoprotein hormones are widely used for various clinical applications, current

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methods for obtaining glycoprotein hormones can be costly, time consuming and/or unable to produce clinically significant quantities of the hormones. For example, hCG is produced for clinical use by
5 purification from pregnancy urine, where it is present in low abundance. Thus, large quantities of pregnancy urine must be collected in order to obtain relatively modest amounts of hCG. Human menopausal gonadotropin (hMG), which is a mixture of partially catabolized
10 human FSH and LH, is extracted from the urine of post-menopausal women, thus entailing the same difficulties in collection as associated with collection of pregnancy urine. Other glycoprotein hormones are even more difficult to obtain in significant quantities due
15 to their low abundance in the biological tissue with which they are associated (e.g., TSH must be isolated from the thyroid, while LH and FSH are found only in the pituitary gland.

Glycoprotein hormones can also be produced
20 as recombinant proteins by expression in eucaryotic cells, such as Chinese Hamster Ovary (CHO) cells. However, production of recombinant glycoprotein hormones in cultured CHO cells is expensive due to the high cost of growth medium, which must contain calf
25 serum for growth of the cultured cells. Moreover, most eucaryotic *in vivo* expression systems are limited in their yield of recombinant gene products, and therefore, are not particularly useful for
manufacturing clinically significant quantities of
30 glycoprotein hormones.

Current methods for producing deglycosylated glycoprotein hormones or hormone subunits, for use and development as antagonists, are also limited. The classic method for deglycosylating the glycoprotein
35 hormones has been by chemical means, e.g., treatment with anhydrous hydrogen fluoride or trifluoromethane

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sulfonic acid. While such methods are capable of removing the N-linked carbohydrates of the α and β subunit, they may produce internal peptide cleavages or changes in secondary structure. See, e.g., Ryan et al., 1988, *supra* at page 2663. Moreover, such methods may not be capable of completely removing N-linked oligosaccharides (for example, anhydrous hydrogen fluoride or trifluoromethane sulfonic acid treatment does not result in the removal of the N-acetylglucosamine coupled to the asparagine residue). See, e.g., Ryan et al., 1988, *supra* at page 2663. Furthermore, such chemical treatments can result in changes in the tertiary structure of the hormone subunit or heterodimer.

More recently, deglycosylated hCG has been produced by *in vitro* mutagenesis of genes encoding the subunits, followed by expression in eucaryotic cells. Dunkel et al., 1993, *supra*. Thus far, these methods have been restricted to the removal of N-linked oligosaccharides from α and β subunits by substituting glutamine for asparagine at the appropriate sites on the respective polypeptides. Although such methods are more reliable for producing N-deglycosylated subunits, they suffer from the same drawbacks as mentioned above relative to high cost and low yield of recombinant glycoprotein hormone production in cultured eucaryotic cells.

From the foregoing discussion, it can be seen that a need exists for a low-cost, simple and reliable method to produce glycoprotein hormones in amounts sufficient for clinical applications. Significant additional advantages would accrue if such methods were capable of producing fully deglycosylated glycoprotein hormones or hormone subunits for use and development as antagonists of native glycoprotein hormones. These needs could be met by producing

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glycoprotein hormones as recombinant products in a procaryotic expression system. Procaryotic systems are well known in the art for their relative ease of use and ability to generate large amounts of gene products. Moreover, since procaryotic cells do not possess the cellular mechanism for glycosylating proteins, procaryotic expression systems would be particularly well suited for producing deglycosylated glycoprotein hormone subunits.

Although procaryotic expression seems an ideal method for producing clinically significant quantities of glycoprotein hormones, such methods have heretofore not been successfully applied to the production of glycoprotein hormones. Previous attempts have produced only aggregated or fragmented subunits, presumably due to the incomplete or incorrect formation of disulfide bonds during folding. In eucaryotic cells, glycoprotein hormones normally form disulfide bonds during folding in the endoplasmic reticulum (ER), under redox conditions that are substantially more oxidizing than found in the bacterial cytoplasm, e.g., of *E. coli*. Wunderlich & Glockshuber, Protein Science, 2: 717-726 (1993). As a result, the disulfide bond formation required for folding of either subunit would not occur in procaryotes such as *E. coli*, such that only unfolded forms of the subunits may be obtainable in procaryotic systems. It is likely that the current unavailability of a procaryotic expression system for producing biologically active recombinant glycoprotein hormones is substantially attributable to these difficulties associated with proper folding of glycoprotein hormone subunits in the procaryotic environment.

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SUMMARY OF THE INVENTION

In accordance with the present invention, methods have been devised to produce biologically active glycoprotein hormones by expressing unfolded glycoprotein hormone subunits in procaryotic cells and re-folding the subunits *in vitro* to form assembly-competent subunits and to produce functional glycoprotein hormones. According to one aspect of the present invention, a glycoprotein hormone subunit is produced in procaryotic cells, which is capable of assembling with a counterpart subunit to form a glycoprotein hormone having at least one activity of a naturally occurring glycoprotein hormone. The invention also provides a recombinant glycoprotein hormone, which comprises an α subunit and a β subunit, at least one of the subunits being produced in procaryotic cells, and the hormone having at least one activity of a naturally occurring glycoprotein hormone.

In one embodiment of the invention, the recombinant glycoprotein hormone comprises one subunit produced in procaryotic cells, and the other subunit (sometimes referred to herein as the "counterpart" subunit) is purified from natural sources. According to a preferred embodiment of the present invention, both subunits are produced in procaryotic cells, and the recombinant hormone is fully deglycosylated. The deglycosylated glycoprotein hormones act as antagonists to naturally-occurring glycoprotein hormones, and are useful as anti-fertility agents and for treatment of conditions involving overproduction of glycoprotein hormones.

According to another aspect of the present invention, a method is provided for making a glycoprotein hormone subunit capable of assembling with a counterpart subunit to form a glycoprotein

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hormone. The method comprises transforming a procaryotic cell with a nucleic acid molecule that comprises a coding sequence for the subunit, operably linked to regulatory sequences for expressing the coding sequence in the procaryotic cell, and expressing the coding sequence in the procaryotic cell to produce an unfolded form of the subunit. The unfolded subunit is then purified from other components of the procaryotic cell. The purified unfolded subunit is folded *in vitro* by dispersing the subunit in a thiol redox buffer having a redox potential of between about -145 and about -200 mV, which enables ordered formation of disulfide bonds, thereby forming an assembly-competent glycoprotein hormone subunit. Methods are also provided for assembling the aforementioned glycoprotein hormone subunits into functional glycoprotein hormones. These methods involve contacting the assembly-competent subunits with each other in the thiol redox buffer, which causes assembly of the subunit to produce the active glycoprotein hormone.

According to another aspect of the present invention, a solution is provided for folding unfolded protein having a disulfide bond in the folded form. This solution comprises a cysteamine/cystamine redox pair at a combined concentration of between about 2 mM and about 10 mM, the ratio of cysteamine to cystamine being about 2:1 and preferably having a pH of about 8.7. The redox potential of such a buffer ranges from between about -145 to about -200 mV. This redox buffer is useful for refolding disulfide-containing proteins, and is particularly useful for refolding unfolded subunits of glycoprotein hormones, most particularly hCG- β .

According to another aspect of the present invention, a pharmaceutical preparation is provided

described hereinabove. In one embodiment, the pharmaceutical preparation comprises recombinant glycoprotein hormones which are appropriately glycosylated such that the hormones may substitute for naturally-occurring glycoprotein hormones in treatment of conditions which can be relieved by increasing the amount of a glycoprotein hormone in a patient. In another embodiment, the pharmaceutical preparation comprises fully deglycosylated recombinant glycoprotein hormones for treatment of conditions which can be relieved by reducing the amount of a glycoprotein hormone in the body or preventing the binding of a glycoprotein hormone to its cellular receptors in the body.

The procaryotically-produced recombinant glycoprotein hormones of the present invention may be produced at low cost, by simple and reliable methods, in amounts sufficient for clinical applications. Additionally, fully deglycosylated glycoprotein hormones produced by the methods of the invention may be used as antagonists of native glycoprotein hormones.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Kinetics of folding the hCG folding intermediate $p\beta 1$ to $p\beta 2$ in various redox buffers. Shown are fluorograms of nonreducing SDS-PAGE separations of the $p\beta 1$ and $p\beta 2$ intermediates. $p\beta 1$ was folded for 5-80 min in the following redox buffers; Fig. 1A: 1.7 mM reduced glutathione, 0.27 mM oxidized glutathione, pH 7.4 (for the 0-min time point, $p\beta 1$ was alkylated in the absence of redox buffer); FIG. 1B: 1.7 mM reduced glutathione, 0.27 mM oxidized glutathione, 17.5 μ M protein disulfide isomerase (PDI), pH 7.4; FIG. 1C: 6.37 mM cysteamine,

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3.63 mM cystamine, pH 8.7; FIG. 1D: 6.37 mM cysteamine, 3.63 mM cystamine, 17.5 μ M PDI, pH 8.7. [14 C]-labelled molecular weight markers are indicated; bovine serum albumin dimer (M_r 132,000), bovine serum albumin monomer (M_r 66,000), chicken egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000), and α -lactalbumin (M_r 14,000).

FIGURE 2. Assembly of hCG subunits in cysteamine/cystamine and glutathione/PDI redox buffers. Fluorogram of a nonreducing SDS-PAGE separation of [35 S]cysteine-p β 2 from the hCG- α /[35 S]cysteine-p β 2 dimer. [35 S]cysteine-p β 2 was incubated with 1 μ M unlabelled urinary hCG- α for times indicated under in the figure. Reactions were performed in either 0.27 mM oxidized glutathione, +/- 17.5 μ M PDI, pH 7.4 or 6.37 mM cysteamine, 3.63 mM cystamine, pH 8.7. The assembly reaction was stopped by the addition of 100 mM iodoacetate, 50 mM Tris-HCl, pH 8.7 followed by ice-cold nonreducing gel electrophoresis buffer. The abbreviations used are: CymSH, cysteamine + cystamine; GSH, reduced and oxidized glutathione; and GSH + PDI, reduced and oxidized glutathione with protein disulfide isomerase.

FIGURE 3. Detection of recombinant hCG- β (rehCG- β) in bacterial cell lysates. Fig 3A: silver stained reducing SDS-PAGE analysis of cell lysates from uninduced B121(DE3) cells that harbored the pET12-hCG- β expression plasmid (lane 1) and from B121(DE3) cells that had been induced with IPTG for 4 hrs (lane 2). Fig 3B: Western blot analysis by enhanced chemiluminescence (ECL, Amersham) of cell lysates from uninduced (lane 1) and induced (lane 2) B121(DE3) cells that contained the pET12-hCG- β plasmid.

FIGURE 4. Purity of rehCG- β during the two step purification. FIG. 4A: Lane 1, silver stain of

a reducing SDS-PAGE analysis of solubilized inclusion bodies from an induced culture of B121(DE3) cells transfected with the pET12-hCG- β plasmid. Lane 2, gel analysis of rehCG- β following C₄ reversed-phase HPLC.

5 Peak fractions were pooled, concentrated, and an aliquot applied to lane 2.

FIGURE 5. Nonreducing SDS-PAGE analysis of forms of HPLC-purified rehCG- β during *in vitro* folding. Lane 1, molecular weight standards: 10 cytochrome C (12.3 kDa), β -lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), and bovine serum albumin monomer (66 kDa). Lanes 2-12, products of rehCG- β folding reactions. Unfolded rehCG- β that had been HPLC purified was diluted to 15 1.28 μ M in the presence of 50 mM Tris-HCl, pH 8.7, 1 mM EDTA, 6.4 mM cysteamine, and 3.6 mM cystamine and incubated at room temperature for the times indicated. Folding was stopped by the addition of iodoacetate and nonreducing gel electrophoresis sample buffer. Gel- 20 resolved forms of folded rehCG- β were visualized by silver staining. " β folding intermediate" is analogous to p β 1 and "folded β " to p β 2 in the intracellular folding pathway.

FIGURE 6. Nonreducing SDS-PAGE analysis at 25 4°C to detect assembly of hCG- α and HPLC-purified rehCG- β subunits. Lane 1, molecular weight standards as in Fig. 5. Lane 2, hCG- α . Lane 3, reduced, carboxy-methylated hCG- β . Lanes 4-13, products of the coupled rehCG- β folding/hCG- α assembly assay. RehCG- β 30 was diluted to 1.28 μ M in the presence of 1 μ M urinary hCG- α and the reaction buffer detailed in the legend of Fig. 5A. Folding and assembly was allowed to occur at room temperature for the times listed in the figure. Reactions were stopped by the addition of 35 iodoacetate and ice-cold nonreducing gel electrophoresis sample buffer. The hCG dimer and

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unassembled subunits were visualized by silver staining.

5 **FIGURE 7.** Nonreducing, 4°C SDS-PAGE analysis of aliquots of anion exchange HPLC fractions for the purification of rehCG- β /urinary α dimer. Lane 1, molecular weight standards as in Fig. 6; remaining lanes, 10% of the indicated HPLC fractions. Bands were visualized by silver staining.

10 **FIGURE 8.** Western blot analysis of the rehCG- β /urinary α hCG dimer. Standard urinary hCG- α and urinary hCG- β subunits and the subunits in the putative recombinant hCG dimer were resolved on reducing SDS-PAGE gels and probed with an antibody to either hCG- α (Fig. 8A) or hCG- β (Figure 8B). Note
15 that rehCG- β migrates faster than urinary hCG- β because the former lacks carbohydrate.

FIGURE 9. Analysis of the *in vitro* receptor binding and activation ability of the recombinant hCG dimer. FIG. 9A: displacement of ^{125}I -hCG binding to
20 hLH receptors by wild-type hCG (+), rehCG- β /urinary α hCG (O), and unfolded rehCG- β (·). Cells derived from a human fetal kidney cell line ("293" cells) were transfected with the hLH receptor expression plasmid pCMX-hLHR, and binding of radiolabelled hCG was
25 measured 20 h later. Cells (2×10^5 /tube) were incubated for 18 h after adding simultaneously ^{125}I -hCG with or without varying doses of wild-type hCG, rehCG- β /urinary α /hCG, or unfolded rehCG- β . Displacement of ^{125}I -hCG is shown as a percentage of maximal binding
30 at each dose of unlabelled hormone. Mean \pm SEM. FIG. 9B: stimulation of cAMP accumulation by wild-type hCG (+), rehCG- β /urinary α hCG (O), or unfolded rehCG- β (·) in 293 cells expressing hLH receptors. Extracellular cAMP accumulation was measured after
35 incubation of transfected 293 cells (2×10^5 cells/tube) for 18 h at 37°C in the absence and

+/- SEM of three experiments.

FIGURE 10. Number of ovulated oocytes (mean \pm SEM) recovered from the oviducts of PMSG-primed rats after injection with purified hCG or recombinant hCG- β /urinary α . Immature hypophysectomized female rats received a subcutaneous priming dose of PMSG (10 IU), followed 52 h later by another subcutaneous injection of varying doses of native hCG or rehCG- β /urinary α . Control animals (C) received no hormone after PMSG priming. At 18-20 h after hCG treatment, the oviducts were excised to determine the number of ovulated ova. The number of rats that ovulated per total number injected is shown at the top of each column.

15 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a biologically active glycoprotein hormone, at least one subunit of which has been produced by expression in a procaryotic cell. The invention also provides methods for expressing glycoprotein hormone subunit-encoding DNA sequences in procaryotes, and for purifying procaryotically-expressed subunits in an unfolded form and re-folding those proteins to produce assembly-competent glycoprotein hormone subunits. Biologically active hormones are produced by assembling the recombinant subunits, either with other recombinant counterpart subunits or with native counterpart subunits. As used herein, the term "counterpart subunit" refers to one or the other of the two non-identical subunits (α or β) comprising a glycoprotein hormone heterodimer. For example, if a β subunit is discussed, its counterpart subunit is an α subunit.

Recombinant glycoprotein hormones produced by the methods described herein possess at least one biological activity of a naturally-occurring

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glycoprotein hormone. These activities include a receptor binding activity and a signal-transducing activity (as defined in the Background section), to elicit the biological response attributable to the respective glycoprotein hormones (e.g., stimulation of cAMP production and *in vivo* stimulation of ovulation). In one embodiment of the invention, the recombinant glycoprotein hormones described herein possess both a receptor binding activity and a signal-transducing activity. In another embodiment, glycoprotein hormones comprising deglycosylated α and β subunits are produced, which possess a receptor binding activating but no signal-transducing activity. These recombinant glycoprotein hormones are useful as antagonists for native glycoprotein hormones.

Sections I-III below set forth details and preferred embodiments for practicing the present invention, including: (1) procaryotic expression and purification of unfolded glycoprotein hormone subunits; (2) re-folding of unfolded subunits to produce assembly-competent subunits and biologically active hormones; and (3) uses of the recombinant glycoprotein hormones to replace naturally-occurring and eucaryotically-expressed glycoprotein hormones in currently available treatments for pathological conditions associated with glycoprotein hormone under expression, and use of recombinant glycoprotein antagonists as anti-fertility agents or for treatment of pathological conditions associated with over-expression of glycoprotein hormones.

In the description that follows, unless otherwise specified, standard cloning and recombinant DNA procedures, such as those described in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") are used. To the extent that other specific materials are

mentioned, it is merely for purposes of illustration and is not intended to limit the invention.

I. **Procaryotic Expression and Purification
of Unfolded Glycoprotein Hormone
Subunits**

In accordance with the present invention, biologically active glycoprotein hormones are produced by expression in a procaryotic system of DNA segments encoding the respective α and β subunits. Any DNA segment encoding an α or β subunit of CG, LH, FSH or TSH, from any species possessing such hormones, is contemplated for use in the present invention. Human cDNAs are preferred, though genes may also be useful in some cases, and cDNAs or genes from non-human species may also be preferred for certain uses, e.g., production of glycoprotein hormones for veterinary use. DNA segments encoding α or β subunits of various glycoprotein hormones are currently known. These include, but are not limited to, CG- α and CG- β from human, primates, horse, hamster and various fishes, including carp; LH- β from human, cow, sheep, pig and rat; FSH- β and TSH- β from human, cow and sheep (see generally, Ryan et al., 1987, *supra*).

In a preferred embodiment, exemplified extensively herein, cDNAs encoding the α or β subunits of hCG are utilized for procaryotic expression. HCG is one of the best characterized glycoprotein hormones, and is known to be capable of substituting for other gonadotropins, such as LH and FSH, for various clinical disorders (see Basic and Clinical Pharmacology, *supra*). However, exemplification of hCG is not intended to preclude the use of other genes or cDNAs, as described above. Furthermore, if a cDNA or gene encoding a particular glycoprotein hormone subunit is unavailable, it may be obtained by a

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variety of methods known in the art. For example, an appropriate cDNA library (e.g., human, in a preferred embodiment) may be screened with heterologous cDNAs or homologous cDNA fragments, if available, or
5 oligonucleotide probes, derived from currently available sequence information. Alternatively, cDNA expression libraries may be screened with antibodies raised against related glycoprotein hormone subunits, according to methods well known in the art.
10 Similarly, genomic libraries may be screened with DNA probes or antibodies. It is comparatively easy to obtain DNA segments encoding a selected glycoprotein hormone subunit by these screening methods, because of the high level of conservation among subunits obtained
15 from different hormones or different species, especially with respect to the α subunit. Concerning the α subunit, it will be appreciated by one skilled in the art that a single α subunit, such as hCG- α may serve as a common subunit for assembly with β subunits
20 from various sources, due to the identity of the α subunit among glycoprotein hormones within a species. For this reason, the α subunit is sometimes referred to herein as a "common α subunit."

Where DNA sequence information is known, a
25 DNA segment encoding a glycoprotein hormone α or β subunit may also be prepared by oligonucleotide synthesis, according to known methods. Long, double-stranded polynucleotides may be synthesized as several smaller segments of appropriate complementarity, and
30 the complementary segments annealed and ligated to construct a DNA segment encoding the desired subunit. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

Glycoprotein hormone subunit-encoding DNA
35 segments may be maintained in any convenient procaryotic cloning vector. Because of the

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comparatively small size of the DNA segments encoding the subunits (e.g., approx. 0.5 kb), numerous vectors are available. In a preferred embodiment, the DNA segments are maintained in a cloning/expression vector suitable for use in a selected procaryotic expression system.

In accordance with the present invention, DNA segments encoding the α or β subunits of selected glycoprotein hormones may be expressed in any procaryotic expression system, many of which are commercially available. Expression systems developed in *E. coli* are preferred for practice of the present invention, inasmuch as *E. coli* is capable of recognizing transcription and translation signals from a wide variety of organisms, and *E. coli* expression systems have been developed for expression of various eucaryotic DNA sequences. Other bacterial (e.g., *Bacillus subtilis*) expression systems may also be useful for practice of the invention, provided that the subunit-encoding DNA sequences are placed under control of appropriate bacterial transcription and translation regulatory regions.

In a preferred embodiment, the *E. coli*/pET expression system is used for expression of glycoprotein hormone subunits. Plasmids and host strains of the pET expression system are commercially available (Novagen, Inc., Madison, WI). Utilizing this system, selected DNA segments may be inserted into the appropriate plasmid under control of procaryotic regulatory sequences, and proteins may be expressed in inclusion bodies of the bacterial cells. Alternatively, the selected DNA segments may be fused to a DNA segment encoding a signal sequence to enable secretion of the expressed recombinant protein into the periplasmic space.

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As described in the Background section, and in greater detail hereinbelow, the intracellular environment of bacteria such as *E. coli* possesses a reducing redox potential that is not conducive to the folding of proteins that normally form disulfide bonds, such as both subunits of glycoprotein hormones. As a result, expression of glycoprotein subunit-encoding DNA segments in bacteria results in the production of unfolded polypeptide, which has been shown to accumulate in inclusion bodies, and may also accumulate in the periplasmic space (in the case of expression/secretion systems). If an expression/secretion system is utilized (by placing the subunit-encoding DNA segment under the direction of a suitable leader sequence), unfolded glycoprotein hormone subunit may be purified from the culture medium according to standard protocols, including various column chromatographic procedures (e.g., HPLC) or affinity purification using antibodies that immunospecifically bind to unfolded α or β subunits.

In some cases, it may be undesirable or ineffective to express glycoprotein hormone subunits under control of a secretion leader sequence. For example, it may be determined that such leaders are ineffective in directing the bulk of the expressed protein to the periplasmic space, such that mature protein accumulates both inside and outside the bacterial cell. Additionally, it may be determined that the presence of a leader sequence, if not properly cleaved during secretion, inhibits the folding of the expressed glycoprotein subunit. Such limitations have been found associated with the expression of hCG- β in *E. coli*, as described in greater detail in Example 2. Accordingly, a preferred embodiment of the invention calls for expressing a glycoprotein hormone subunit in *E. coli* inclusion

inclusion bodies according to the following methods.

5 First, inclusion bodies are separated from the remainder of components of the *E. coli* cells and culture medium, according to known methods. For example, as described in greater detail in Example 2 below, inclusion bodies containing unfolded hCG- β

10 bacterial cells are pelleted and lysed in an isotonic lysis buffer comprising lysozyme and EDTA, to form protoplasts, which are pelleted, resuspended and homogenized at least once in a detergent-containing buffer, such as 1% Triton X-100. The final

15 homogenization step should be performed in a buffer containing deoxycholate. In accordance with the present invention, we have determined that deoxycholate selectively solubilizes lipids while leaving most of the hCG- β in the inclusion bodies,

20 thus affording a high degree of purification in the solubilization step. Inclusion bodies are then recovered by centrifugation and washing and are solubilized in a buffer containing, e.g., 50 mM Tris-HCl (pH 8.7), 8 M urea, and 50 mM dithiothreitol (DTT)

25 at 37°C for one hour under argon, to prevent oxidation. Unfolded β subunit is purified from solubilized inclusion bodies by reversed-phase HPLC column chromatography, as described in greater detail in Example 2. Fractions containing the subunit are

30 pooled and concentrated, to yield milligram quantities of unfolded hCG- β . For example, the yield of hCG- β in this system was approximately 6-7 mg/l from a 200 ml starting culture and ~25 mg/l from a 2.5 l starting culture, as compared to 0.1 mg/l average yield in

35 commonly-used eucaryotic expression systems.

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Unfolded glycoprotein hormone subunits (α or β) prepared by the methods described above for hCG- β may be used immediately for re-folding and assembly into biologically active glycoprotein hormones, as described hereinbelow. Alternatively, the unfolded protein may be stored at -70°C for a brief time (i.e., 1-2 days), and then used for refolding and assembly of hormones.

Although the expression and purification of hCG- β has been described and exemplified hereinabove, it will be appreciated by those of skill in the art that the same expression and purification techniques can be applied to any of the glycoprotein hormone subunits, due to their strong similarities in structure and physical properties. Of course, the same methods for purifying inclusion bodies comprising any of the glycoprotein hormone subunit may be utilized. Additionally, the conditions described in Example 2 for HPLC purification of hCG- β from solubilized inclusion bodies may be applied to the purification of subunits of other glycoprotein hormones, with minimal modifications. Additionally, these HPLC methods may also be used to purify secreted glycoprotein hormone subunits from culture medium, if an expression-secretion system is used for producing the proteins.

II. Preparation of Assembly-Competent Glycoprotein Hormone Subunits and Biologically-Active Glycoprotein Hormones

As described above, the expression of recombinant disulfide-containing proteins in *Escherichia coli* may result in the formation of inclusion bodies composed of aggregated protein with non-native disulfide bonds. Proteins that normally

form disulfide bonds during folding in the endoplasmic reticulum (ER) are particularly susceptible to this type of aggregation in *E. coli* due to the more reducing potential of the bacterial cytoplasm (Wunderlich & Glockshuber, 1993, *supra*) compared to that of the ER of mammalian cells (Huang et al., Science 257: 1496-1502, 1992). Thus, a challenge in the field of biotechnology is to fold these recombinant proteins *in vitro* while allowing correct disulfide bond formation. However, no standardized *in vitro* conditions have been found that support the oxidative folding of proteins *in vitro* (Fischer et al., Biotechnol., Bioeng. 41: 3-13, 1993).

Many oxidation conditions for refolding proteins have been reported, such as air oxidation and oxidation with chemical reagents such as oxidized glutathione, oxidized DTT, cystine, and cystamine. Disulfide bond formation during protein folding has also been facilitated by proteins such as mammalian protein disulfide isomerase (PDI), which does not seem to change the folding pathways, and by PDI from *E. coli* (DsbA). Moreover, PDI and DsbA have been shown to be required for disulfide bond oxidation in cells (Bardwell et al., Cell 67: 581-589, 1991; Bulleid et al., Nature 335: 649-651, 1988).

As mentioned, significant sequence homology exists among the β subunits of the glycoprotein hormones, including conservation of 12 cysteines that comprise six disulfide bonds in these proteins. Common to each hormone, biological activity depends on assembly of the α and β subunits. We have previously found that the rate-limiting event in subunit assembly in cultured cells is the folding of hCG- β (Ruddon et al., J. Biol. Chem., 262: 12533-12540, 1987). We have also found that folding of hCG- β to an assembly-competent conformation requires the formation of

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specific disulfide bonds, and we have described a folding pathway of this protein based on the order of formation of the 6 disulfide bonds. Importantly, this same pathway has been identified in two different cultured cell lines (Bedows et al., J.Biol.Chem. 267: 8880-8886, 1992; Huth et al., J. Biol. Chem., 267: 21396-21403, 1992a) as well as *in vitro* (Huth et al., J.Biol.Chem. 268: 16472-16482, 1993). The folding pathway can be summarized as follows, where cysteines that become disulfide-linked in a particular transition of the folding pathway are indicated.

<u>hCG Residue</u>		<u>Transition</u>	
	34-88	p β 0	→ p β 1-early
	38-57	p β 1-early	→ p β 1-late
15	9-90, 23-72	p β 1-late	→ p β 2-free
	93-100	p β 2-free	→ p β 2-combined-early
	26-110	p β 2 combined-early	→ p β 2-combined-late

The rate-limiting event in the folding pathway is the conversion from p β 1-late to p β 2-free, which results in an assembly-competent conformation of this subunit. We have determined the $t_{1/2}$ for this folding event to be 4 min in cultured cells (Bedows et al., 1992, *supra*) and 25-80 min *in vitro* (Huth et al., 1993, *supra*). *In vitro*, a $t_{1/2}$ of 25 min was observed when protein disulfide isomerase in a glutathione redox buffer catalyzed the folding of reduced and urea-denatured hCG- β , whereas the slower *in vitro* rate was observed in the glutathione redox buffer alone (Huth et al., 1993, *supra*). Protein disulfide isomerase in a glutathione redox buffer was also found to catalyze the assembly of p β 2-free with hCG- α by a mechanism that involves reduction of the carboxyl-terminal disulfide bonds of hCG- β (Huth et al., 1993, *supra*).

The folding constraints described hereinabove for hCG- β are expected to be equally applicable to both α and β subunits of the other glycoprotein hormones, due to the high conservation of disulfide bonds among the various hormones. This expectation is even further supported by a recent report of the crystallographic structure of intact hCG (α/β dimer), which showed that both the α and β subunits possess a common disulfide-bonded "cystine knot" motif (Lapthorn et al., Nature, 1994; in press). Accordingly, it is extremely likely that other α and β subunits will follow the same re-folding pathway as described above for hCG- β .

Protein folding of glycoprotein hormone subunits *in vitro* requires reagents that support disulfide bond formation but that do not interfere with the normal protein folding process. The mechanism for disulfide formation *in vivo* is thought to occur via a mixed disulfide intermediate involving a protein thiol group and an external thiol such as in glutathione or in PDI. A second protein thiol attacks this mixed disulfide bond (Wetlauer, Meth. Enzymol. 107: 310-302, 1984). Ideally, during oxidative folding, the rate of forming the mixed disulfide does not lag behind the folding rate that positions two cysteines in proximity for a disulfide bond to form. In the case of ribonuclease A, the rate of forming mixed disulfide bonds was limiting in refolding this protein *in vitro* (Rothwarf & Scheraga, Biochemistry 32: 2690-2697, 1993). Furthermore, if a mixed disulfide bond with an external oxidizing agent forms too slowly, both cysteine thiols may become buried in the interior of the folding protein and become inaccessible to the external oxidant. Such an occurrence has been found to retard the folding pathway of bovine pancreatic trypsin inhibitor (Weissman &

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Kim, Cell 71: 841-851, 1992). On the other hand, if two protein thiols form mixed disulfide bonds with external oxidants, the formation of an intramolecular S-S bond will be hindered and folding may be slowed.

5 To prevent oxidation of both cysteines, excess reducing agent is normally present in the redox buffer. As these examples show, the interplay of disulfide chemistry with protein conformational changes can determine how rapidly a protein folds.

10 As described hereinbelow and in Examples 1 and 2, we have formulated redox buffers capable of enabling disulfide bond formation without interfering with the normal folding process of glycoprotein hormone subunit proteins. These buffers support *in*
15 *vitro* folding of glycoprotein hormone subunits at rates approaching *in vivo* folding rates. In addition to being excellent folding buffers for glycoprotein hormone subunits, these redox buffers may be used to advantage as general reagents for *in vitro* folding for
20 disulfide-containing proteins, since any disulfide-containing protein is subject to the same constraints as those described above for glycoprotein hormone subunits.

The redox buffers used for glycoprotein
25 hormone subunit refolding in accordance with the present invention were designed to mimic conditions in the endoplasmic reticulum of mammalian cells, where these subunits normally fold. Accordingly, we have determined appropriate redox potentials, molarities,
30 reagents and additives for these redox buffers, which enable folding of assembly-competent glycoprotein hormone subunits, and assembly of biologically active glycoprotein hormones.

35 Numerous reagents and oxidizing conditions for refolding proteins have been reported, such as reduced and oxidized glutathione, reduced and oxidized

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DTT and cysteamine/cystamine, among others. Protein disulfide isomerase (PDI) from mammals or bacteria (i.e., DsbA) have also been found to facilitate folding of disulfide-containing proteins. To the extent that these reagents are mutually compatible, they and other similar reagents are contemplated for use in the present invention. The redox buffers of the invention generally comprise a redox pair, such as oxidized and reduced glutathione or cysteamine/cystamine. The redox pair should be adjusted in concentration and ratio to achieve a redox potential of between about -145 to -200 mV, which is the optimal range for re-folding of hCG- β , and is also the expected optimal range for the homologous β subunits as well as the common α subunit, since they all possess a common disulfide-bonded "cystine knot" motif, as elucidated by x-ray crystallography (Lapthorn et al., 1994, *supra*).

In a preferred embodiment of the invention, the cysteamine/cystamine redox pair is utilized in a simple, inexpensive thiol folding buffer. Cysteamine/cystamine redox buffers are described in detail in Examples 1 and 2, in relation to *in vitro* folding of chemically unfolded or bacterially expressed HCG- β . The total concentration of cysteamine/cystamine in such a redox buffer should be between about 2 mM and about 10 mM. For optimum *in vitro* folding, the cysteamine/cystamine redox buffer should be maintained at an alkaline pH, preferably at about pH 8.7. The cysteamine/cystamine redox pair may be disposed in any buffer capable of adequate buffering around the desired pH. For example, in a preferred embodiment, 50 mM Tris-HCl, pH 8.7, is utilized. Thus, a preferred thiol redox buffer for use in refolding bacterially expressed glycoprotein hormone subunits comprises, e.g., 6.4 mM cysteamine

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and 3.6 mM cystamine in 50 mM Tris-HCL, pH 8.7. It will be appreciated by those skilled in the art that this redox buffer may be varied to suit slight differences in refolding parameters associated with other glycoprotein hormone subunits, but that such adjustments are merely for optimization of refolding rates.

If glycoprotein subunits are being prepared for later assembly, the thiol redox buffer may be modified by adding 2 M urea, which, in accordance with the present invention, has been found to facilitate folding efficiency (see Example 2). Urea should be removed by dialysis or similar dilution methods prior to using the folded subunit for assembly into heterodimers.

In an alternative embodiment, a thiol redox buffer comprising reduced and oxidized glutathione is utilized for refolding glycoprotein hormone subunits. Preferably, the glutathione redox buffer also contains protein disulfide isomerase (PDI), which has been found to accelerate folding rates of the glycoprotein hormone subunits in glutathione redox buffers (See Examples 1 and 2). A glutathione/PDI redox buffer may be preferred in some instances, since it may promote somewhat more efficient assembly of subunits than the cysteamine/cystamine redox buffer under certain conditions (See Example 1). However, inclusion of PDI in the redox buffer increases the cost of such buffers, and is therefore not preferred for large-scale refolding reactions, such as those that may be employed for production of commercially significant quantities of glycoprotein hormones.

Glutathione redox buffers should comprise a combined concentration of oxidized and reduced glutathione of about 2mM and a ratio of reduced to oxidized glutathione of approximately 6 to 1.

Glutathione redox buffers are preferably maintained at a neutral or slightly alkaline pH, e.g., about pH 7.4, in an appropriate buffer, such as 50 mM sodium phosphate (though a pH of as high as 8.7 may be used). Thus, an example of a preferred glutathione buffer for use in the present invention comprises 1.7 mM reduced glutathione, 0.27 mM oxidized glutathione and 17.1 μ M PDI (i.e. 1mg/ml) in 50 mM sodium phosphate, pH 7.4. It will be appreciated by those skilled in the art that the glutathione redox buffer may be modified to optimize refolding of different glycoprotein hormone subunits.

The cysteamine/cystamine and glutathione redox buffers described hereinabove can be used for small- and large-scale *in vitro* refolding reaction involving bacterially expressed unfolded glycoprotein hormone subunits. Both of the aforementioned redox buffers were designed to mimic the redox conditions found in the eucaryotic endoplasmic reticulum, and to effect a redox potential of between -145 and -200 mV. Additionally, these and similar buffers are useful for folding other disulfide-containing proteins, as described hereinabove.

Procaryotically expressed glycoprotein hormone subunits are folded into assembly-competent conformations and assembled into biologically active glycoprotein hormones in the above-described thiol redox buffers. To accomplish this, an appropriate concentration of unfolded subunit is added to an aliquot of buffer, and incubated at a suitable temperature (i.e. 22-28° C) for a pre-determined amount of time to enable the subunits to fold. In accordance with the invention, it has been discovered that *in vitro* folding of glycoprotein hormone subunits is facilitated by adding unfolded subunit to redox buffer at dilute concentrations of the protein (e.g.,

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0.02 to 0.05 mg/ml). Example 2 describes in detail the re-folding of bacterially-expressed hCG- β in the buffers, and under the conditions, described hereinabove. Typically, refolding reactions were
5 incubated for 1 min - four hours at room temperature, although higher temperatures and concomitantly different incubation times, may be utilized, if desired.

The folding reactions described hereinabove
10 result in the formation in assembly-competent glycoprotein hormone subunits, which may be used immediately in assembly reactions, or which may be stored at -70° C for future use.

Biologically active glycoprotein hormones
15 are assembled from recombinant and/or native subunit in a variety of ways. In one embodiment, a bacterially-expressed subunit is refolded in the presence of a naturally-occurring counterpart subunit, thereby producing the biologically active heterodimer
20 within a single refolding reaction. This embodiment is described in Example 2, wherein bacterially-expressed hCG- β is folded and assembled with urinary hCG- α .

In an alternative embodiment, both the α and
25 the β subunits may be folded separately and combined in the thiol redox buffer for assembly into a biologically active glycoprotein hormone. In yet another embodiment, unfolded α and β subunits may be combined in the same refolding reaction, whereby both
30 subunits are folded and assembled in that reaction.

Biologically active recombinant heterodimers produced as described above may be purified from the reaction mixture by standard methods, such as HPLC or conventional ion exchange chromatography. For
35 instance, as described in Example 2, a recombinant hCG- β/α dimer was purified from the reaction medium by

injection onto a DEAE column followed by elution in a 0-1 M NaCl gradient in a neutral phosphate buffer over 100 minutes.

After biologically active glycoprotein hormones have been purified from the refolding medium, they may be tested for biological activity by *in vitro* and *in vivo* assays known in the art. For example, receptor binding activity may be tested in a *in vitro* assay by binding to the CG/LH receptor in appropriate culture target cells, such as the human fetal kidney cell line, designated "293" cells, transfected with the CG/LH receptor gene (Dunkel et al., 1993, supra). These same cultured cells may be utilized for *in vitro* assays of signal transduction activity, by assaying for the ability of the recombinant glycoprotein hormones to stimulate cAMP production in "293" cells (See, e.g., Dunkel et al, 1993, supra). The use of these two *in vitro* assays is described in Example 2, wherein it was shown that a heterodimer comprising bacterially expressed hCG- β /urinary hCG- α was capable of binding to the CG/LH receptors of 293 cells, and was further capable of stimulating cAMP production in those cells.

Recombinant glycoprotein hormones may also be tested for biological activity in *in vivo* assay systems. For instance, as set forth in Example 2 herein, the recombinant hormone produced as described in Example 2 was tested for the ability to stimulate ovulation upon injection into mice. This *in vivo* assay demonstrated that the recombinant hCG described in Example 2 was capable of exerting this *in vivo* biological activity, which implied its ability to bind to appropriate receptors *in vivo* and to exert a signal transduction activity.

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III. METHODS OF USING PROCARYOTICALLY PRODUCED GLYCOPROTEIN HORMONES

The recombinant glycoprotein hormones described hereinabove can be used for any of the pharmaceutical purposes for which naturally-occurring and eucaryotically expressed glycoprotein hormones are currently used. These uses include, but are not limited to: stimulating ovarian follicle development and ovulation in women, inducing spermatogenesis and testosterone production in men, or for treatment of other forms of hypogonadism or conditions associated with gonadotropin or TSH deficiencies. Dosages of glycoprotein hormones for treatment of these various conditions in men and women are well developed, as set forth, for example, in Basic and Clinical Pharmacology, *supra*. Once the potency and biological activity of the recombinant proteins described herein have been determined, dosages can be standardized by International Unit (IU), according to methods well known to medicinal chemists.

The recombinant glycoprotein hormones produced in accordance with the present invention may also be used for various veterinary purposes. For example, equine CG or bovine, equine or porcine LH may be produced in bacteria and used to induce ovulation, spermatogenesis, etc., in the respective animals. Accordingly, to the extent that the term "patient" is used herein, it refers to both humans and animals.

In another embodiment, fully deglycosylated recombinant glycoprotein hormones produced according to the methods of the invention may be used and developed for use as antagonists to native glycoprotein hormones. As described hereinabove, such fully deglycosylated hormones have been found to act as antagonists to naturally occurring hormones. Accordingly, such hormones may be utilized as anti-

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fertility agents or for treatment of pathological conditions associated with overproduction of certain of the glycoprotein hormones, (e.g., hyperthyroidism).

5 Finally, it will be appreciated by those skilled in the art that, if desired, procaryotically produced glycoprotein hormone subunits may be re-glycosylated by various chemical or enzymatic means (e.g., via glycosyl transferases). Re-glycosylated subunits may then be used as described above to
10 produce glycoprotein hormones with signal transduction activity.

The following examples are provided to describe the invention in greater detail and to present preferred embodiments of the invention. These
15 examples are intended to illustrate, not to limit, the invention.

EXAMPLE 1

20 Redox Conditions for Stimulating of *in vitro* Folding and Assembly of the Glycoprotein Hormone Chorionic Gonadotropin

In this Example, we present the results of a comparison of redox conditions involving cysteamine, cystamine, oxidized and reduced glutathione, and mammalian protein disulfide isomerase in order to
25 identify a redox buffer composed of low molecular weight components that is as efficient as protein disulfide isomerase in supporting the oxidative folding of a model substrate, the β subunit of human chorionic gonadotropin (hCG- β). The intracellular
30 folding pathway of this protein has been characterized, as described in the Detailed Description (Huth et al., 1992a, *supra*; Huth et al., J.Biol.Chem. 267: 2870-8879, 1992b, Ruddon et al., 1987, *supra*), allowing a direct comparison of *in vitro*
35 folding rates to that which normally occurs in cells.

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5 In this way, we have identified optimal *in vitro* hCG- β folding conditions by employing two types of oxidizing reagents that have both been reported to be the major oxidant in the endoplasmic reticulum (ER) of mammalian cells: glutathione and cystamine.

10 In the results that follow, we show that in a buffer composed of cysteamine and cystamine, the same rate of folding p β 1-late to p β 2-free (see Section II in the Detailed Description) and of assembly of p β 2-free with hCG- α occurs as in the presence of protein disulfide isomerase in an optimal glutathione redox buffer. Furthermore, the rates of folding and assembly of hCG subunits in this cysteamine/cystamine redox buffer approach the rates of these processes
15 that normally occur in intact cells.

MATERIALS AND METHODS

Materials. [35 S]cysteine-p β 1 and [35 S]cysteine-p β 2 were obtained from metabolically labelled JAR choriocarcinoma cells as described
20 previously (Huth et al., 1993, *supra*). Urinary hCG- α was obtained from Dr. Gabriel Bialy from the National Institutes of Health. Bovine liver protein disulfide isomerase was purchased from Takara Biochemical (Palo Alto, CA) and diluted with water to 10 mg/ml (10X stock) for use in folding and assembly assays.
25 Recombinant rat liver PDI was also used in assembly assays and did not differ in efficacy of catalyzing assembly of hCG α and β subunits. Cystamine and cysteamine were purchased from Aldrich Chemical Co.
30 (Milwaukee, WI). Stock 100 mM solutions of each were prepared in 10 mM HCl within 30 min of the *in vitro* assay. 10X stock solutions consisted of 17.3 mM cysteamine and 2.7 mM cystamine, or 63.7 mM cysteamine and 36.3 mM cystamine. All 10X redox buffers were
35 prepared in 10 mM HCl to slow the rate of air

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oxidation. After dilution to 1X, the value of [reductant]²/[oxidant] was maintained at 11.1 mM in both the 2 mM and 10 mM buffers (final concentration). Glutathione redox buffers were prepared similarly and the final concentrations of reduced and oxidized glutathione in the 2 mM or 10 mM redox buffers were the same as the concentrations of oxidant and reductant in the cysteamine/cystamine buffers. The standard redox potentials of cysteamine and glutathione have been reported to be nearly the same (Keire et al., J.Org.Chem. 57: 123-127, 1992). As a result, the redox potential of both types of redox buffers did not vary in the conditions used in the experiments reported here. Given that the standard redox potential of glutathione is between 0.205 v and 0.26 v, the redox potential of the buffers used in the experiments reported here was calculated to be between -145 and -200 mV. We have varied the value of [reductant]²/[oxidant] and found optimum folding of hCG- β to occur between values of 2 and 40 mM (Huth and Ruddon, unpublished data). Iodoacetate, sodium salt was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was diluted in 450 mM Tris-HCl to a concentration of 900 mM. The water used in these experiments was millipore filtered, filter-degassed and purged with argon for 15 min prior to use.

***In vitro* hCG- β folding and assembly assays.** 2500 CPM of [³⁵S]cysteine-labelled p β 1 (approximately 50 pg) was purified from JAR choriocarcinoma cells as previously described (Huth et al., 1993, *supra*) and was added to a 40 μ l reaction consisting of 50 mM Tris-HCl, pH 8.7 or 50 mM sodium phosphate buffer, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and, where indicated, 17.5 μ M PDI. The redox buffer consisted of 2 mM or 10 mM of cysteamine + cystamine or reduced glutathione + oxidized glutathione.

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Eppendorf tubes that contained these reactions were flooded with argon and then incubated at 37°C. Folding was stopped by the addition of 5 μ l of 900 mM iodoacetate in 450 mM Tris-HCl. After 5-10 min of alkylation, samples were mixed with 50 μ l of nonreducing gel electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 40 μ g bromophenol blue). The buffer composition of the assembly reactions was identical to that of the folding reactions. In these assays, fully oxidized [³⁵S]cysteine-p β 2 was incubated with 1 μ M hCG- α . Assembly was allowed to occur for times indicated in the description of the drawings and was stopped by the addition of 5 μ l 900 mM iodoacetate in 450 mM Tris-HCl. After 5 min. 50 μ l of ice-cold sample buffer was added to the reaction, and the samples were stored at -4 to -20°C before gel analysis.

Folding intermediates and unassembled versus assembled p β 2 forms were purified on nonreducing SDS-PAGE gels and visualized by fluorography as described (Huth et al., 1993, *supra*).

Quantitation of the rates of folding and assembly. The percent folding at each time point was determined by comparing the amount of [³⁵S]cysteine in the p β 2 and p β 1 loci on the gels (Ruddon et al., 1987, *supra*). Folding of hCG- β was assumed to be first order, and half-times of folding were determined from the initial rates of folding as shown on semilog plots (Huth et al., 1993, *supra*). Similarly, the half-times of hCG- β subunit assembly could be determined by quantitating the amount of unassembled p β 2 and p β 2 in the hCG dimer. Although assembly is a second order process, since hCG- α is in ~5000-fold excess in our reactions, the reaction is pseudo-first order. The half-time of assembly could also be determined from

the slopes of semi-log plots as described (Huth et al., 1993, *supra*).

EXPERIMENTAL RESULTS

in vitro Folding of hCG- β

5 The hCG- β folding pathway that is summarized above (Detailed Description, Section II) includes 5 transitions that involve disulfide bond formation during tertiary and quaternary structure formation in this hormone. Here we show the results *in vitro*
10 assays in which steps 3 and 4 were reconstituted under various redox conditions. Step 3 is the rate limiting event in the folding of hCG- β and results in a conformation of hCG- β that can assemble with hCG- α . Because this folding event is slow, requires specific
15 redox conditions, and can be inhibited by detergents, we are able to purify the [35 S]cysteine-p β 1-late intermediate from metabolically labelled, detergent-solubilized JAR cells and store it at acidic pH for use in refolding reactions. In the remainder of this
20 report, p β 1-late will be referred to as p β 1 for simplicity. Step 4 refers to the assembly of the hCG subunits where the carboxyl-terminal disulfide bonds of hCG- β are reduced. This is required for efficient assembly to occur (Huth et al., 1993, *supra*). The
25 assembly reaction can be reconstituted *in vitro* by purifying completely oxidized [35 S]cysteine-p β 2-free from cells. During the assembly reaction *in vitro*, redox reagents enhance the rate of assembly by facilitating the isomerization of the carboxyl-
30 terminal disulfide bonds of p β 2-free. In the rest of this report, we refer to p β 2-free simply as p β 2.

 The intermediates involved in these *in vitro* folding reactions, p β 1, p β 2, and dimer hCG, have all been distinguished by antibody reactivity, migration
35 on C_4 reversed-phase HPLC, migration on nonreducing

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SDS-PAGE gels, and by peptide mapping on reversed-phase HPLC. In the experiments reported here, the efficiency of folding and assembly was monitored by shifts among the $p\beta 1$ and $p\beta 2$ intermediates and the hCG dimer on nonreducing SDS-PAGE gels.

Shown in Figure 1 are examples of the $p\beta 1$ to $p\beta 2$ folding reaction that illustrate the differences that we have identified while folding hCG- β under various redox conditions. In these experiments, approximately 50 pg of the [^{35}S]cysteine- $p\beta 1$ folding intermediate was incubated in oxidized and reduced glutathione or cysteamine/cystamine redox buffers, with or without 1 mg/ml PDI, for times ranging from 5-80 min. $p\beta 1$ was separated from $p\beta 2$ on nonreduced SDS-PAGE gels, and the bands were visualized by fluorography (Ruddon et al., 1987, *supra*). The first observation is that PDI can catalyze the folding of $p\beta 1$ as was demonstrated in our earlier report (Huth et al., 1993, *supra*) (compare Figure 1A to 1B). Furthermore, the choice of the type of redox buffer can markedly affect the rate of $p\beta 1$ folding. A comparison of Figures 1A and 1C shows how the folding rate is faster in a cysteamine/cystamine redox buffer than in a glutathione redox buffer. We also found that under some redox conditions, PDI could inhibit the folding of $p\beta 1$ by forming stable complexes with $p\beta$ forms (Figure 1D). Finally, different heterogeneity was found in the $p\beta 2$ locus depending on the redox conditions used. We have found that in the glutathione redox buffers, a heterogeneous population of $p\beta 2$ results (Figure 1B) that differs in the extent of oxidation of the carboxy-terminal disulfide bonds (Huth and Ruddon, unpublished data). However, a more homogeneous population of $p\beta 2$ formed in the cysteamine/cystamine redox buffer (Figure 1C).

Optimal Folding Conditions for hCG- β

Because the different redox conditions resulted in different efficiencies of folding hCG- β , a parallel set of conditions involving cysteamine and cystamine or oxidized and reduced glutathione with and without PDI was tested in this hCG- β folding reaction in order to find optimal conditions for each redox buffer. Shown in Table 1 is summary of the initial rates of folding p β 1-late to p β 2-free and the efficiency of these reactions in the glutathione or cysteamine/cystamine redox buffers, where the total amount of oxidant and reductant was 2 or 10 mM and the pH either 7.4 or 8.7. Also shown in this table is the effect of 1 mg/ml PDI under each of these redox conditions.

In the 2 mM glutathione redox buffer in the absence of PDI, the initial rate was faster at pH 8.7 although the efficiency of folding to p β 2 was diminished. Increasing the concentration of the redox buffer from 2 mM to 10 mM, while maintaining the same redox potential, did not significantly improve the rate or efficiency of folding. In all of the glutathione buffers tested, PDI increased the rate of folding. However, the efficiency of folding was limited in the presence of PDI due to formation of a stable complex between [35 S]cysteine-labelled p β forms and PDI that was detected on nonreducing gels (see Figure 1). A greater proportion of p β forms complexed with PDI in the pH 8.7 buffers. Overall, the glutathione reaction condition that resulted in the best rate and efficiency of folding of p β 1 was 2 mM glutathione at pH 7.4 in the presence of PDI.

TABLE 1

Comparison of oxidized and reduced glutathione and cysteamine/cystamine redox buffers in the *in vitro* hCG- β folding assay. The amounts of oxidant and reductant in the 2 and 10 mM redox buffers are listed in the Materials and Methods as is the procedure for the folding reaction. Where indicated, PDI was included in the assay at 1 mg/ml (17.5 μ M)

Redox reagent ^a	Amt (mM)	pH	-PDI		+PDI		% complex ^c
			$t_{1/2}$ (min) ^b	Final percent folding ^c	$t_{1/2}$ (min) ^b	Final percent folding ^d	
GSH	2	7.4	79	54	21	58	14
	10	8.7	35	39	16	50	24
		7.4	70	40	24	50	15
CymSH	2	8.7	ND ^e	28	20	37	46
		7.4	46	55	20	45	27
	10	8.7	30	64	ND ^e	17	61
		7.4	24	75	26	47	23
		8.7	16	72	21	49	31

^a Abbreviations used are: GSH, oxidized + reduced glutathione; CymSH, cysteamine + cystamine.

^b Half times for folding reactions were calculated from the initial rates on semilog plots.

^c The "final percent folding" indicates the amount of folding after 80 minutes in the redox buffers that lacked PDI. At this time, the percent folding was approaching steady state, which was less than 50% in some cases. The data shown are the mean of two or more experiments that did not differ by more than + 10% from the mean.

^d The "final percent folding" in the PDI-containing reactions indicates the percent p β 2 calculated from the ratio (p β 2)/(p β 1 + p β 2 + p β -PDI complex), after 40 min of incubation. At this time, the percent folding was close to the maximum (data not shown). These experiments were carried out twice; once to determine the optimal redox conditions in the presence of 1 mg/ml PDI and then repeated under optimal conditions.

^e The percent complex indicates the amount of [³⁵S]cysteine-p β forms found in the PDI complex on nonreducing SDS-PAGE gels. The amount of p β forms in this complex did not seem to vary over the incubation times tested (data not shown).

^f ND = not determined because the kinetics were not first order.

In cysteamine/cystamine redox buffers, p β 1 folding rates were ~2-fold faster than those observed in analogous glutathione redox buffers, and in both redox buffers p β 1 folded faster at pH 8.7 than at pH 7.4. PDI increased the rate of hCG- β folding at pH 7.4 in the 2 mM cysteamine/cystamine redox buffer, but had no positive effect on the rate under the other conditions. Furthermore, in all of the cysteamine solutions tested, PDI bound to 20-60% of p β forms resulting in a lower folding efficiency. Complex formation was particularly apparent at pH 8.7. Overall, the optimal rate of folding of hCG- β occurred at pH 8.7 in the presence of 10 mM cysteamine + cystamine, without PDI.

In comparing the redox conditions shown in Table 1, we found that the optimal cysteamine/cystamine buffer and the optimal glutathione/PDI buffer resulted in similar rates of hCG- β folding. In two independent experiments, the half-time of folding p β 1 to p β 2 in the optimal glutathione/PDI redox buffer was 20-22 min (Table 1). This rate is similar to the 25 min half-time reported for the folding of reduced and urea-denatured hCG- β in the presence of this glutathione/PDI buffer (Huth et al., 1993, *supra*). In three kinetic studies using the optimal cysteamine/cystamine redox buffer, we found that the half-time of folding was 16 +/- 4 min (Table 1). These *in vitro* rates of the p β 1 to p β 2 folding event are only 4- to 5-fold slower than the rate observed in cells (Ruddon et al., 1987, *supra*).

***In vitro* Assembly of hCG- β and hCG- α**

We have previously shown that PDI not only catalyzes the oxidative folding of hCG- β but that this enzyme also catalyzes the assembly of fully oxidized hCG- α and β subunits (Huth et al., 1993, *supra*). The

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mechanism of PDI-catalyzed assembly seems to involve a reduction of the carboxy-terminal disulfide bonds of hCG- β that results in an assembly competent conformation of this subunit. As another test of the ability of the cysteamine/cystamine redox buffer to support the production of hCG, the *in vitro* assembly of [35 S]cysteine-p β 2 with urinary hCG- α was tested with this mixture of reagents. Shown in Figure 2 is an example of this assay where the unassembled p β 2 subunit was separated from the α -p β 2 dimer on nonreducing SDS-PAGE gels at 4°C. The amount of [35 S]cysteine-labelled hCG- β in each gel band was determined as described in the Materials and Methods. The half-time for assembly in the cysteamine/cystamine redox buffer, estimated from the initial slopes of semilog plots of the extent of folding versus time, was found to be 12 min. This rate is comparable to the 10-min half-time observed in the PDI-catalyzed assembly reaction (Figure 2) and comparable to the 8-min half-time observed *in vivo* (Bedow et al., 1992, *supra*). In the glutathione redox buffer that lacked PDI, assembly was inefficient (Figure 2). This is consistent with our earlier studies that showed the requirement for overnight incubations in the glutathione redox buffer alone to achieve significant assembly (Huth et al., 1993, *supra*). While the rates of assembly in the cysteamine/cystamine and glutathione/PDI buffers were similar, the efficiency of assembly was found to be greater in the glutathione/PDI buffer. For example, after 15 min of incubation, the extent of assembly reached a plateau at 60% in the cysteamine/cystamine redox buffer, whereas in the glutathione/PDI buffer, 80% assembly was achieved after a 60 min incubation.

The results of these folding and assembly assays indicate that the production of complete hCG

can be optimized by a simple buffer system composed of cysteamine and cystamine at alkaline pH.

EXAMPLE 2

Folding and Assembly into a Functional $\alpha\beta$ Dimer of a Bacterially Expressed hCG- β

In this Example, we demonstrate the expression of unfolded hCG- β in the inclusion bodies of *E. coli*, the purification of this unfolded protein, and in vitro folding and assembly. We also demonstrate biological activity of an hCG dimer composed of nonglycosylated rehCG- β and glycosylated urinary hCG- α .

I. MATERIALS AND METHODS

a. Reagents

A plasmid containing the cDNA for the human hCG- β gene (sequence disclosed by Fiddes & Goodman; Nature 286: 684-687, 1980) was obtained from Dr. Fiddes. Purified hCG- α was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH. Cysteamine and cystamine were obtained from Aldrich chemical corporation (Milwaukee, WI). For expression of recombinant hCG- β , the pET system (Novagen, Madison, WI) was used. Isopropyl- β -D-thiogalactopyranoside (IPTG) (United States Biochemical Corporation, Cleveland, OH) was used to induce protein expression in B121 (DE3) cells (Novagen, Madison, WI).

b. Construction of the pET Expression Plasmid Containing the hCG- β Gene

To prepare the hCG- β cDNA for ligation into the pET-12a vector, the polymerase chain reaction was employed. The 5' sense primer was designed to remove the coding region for the mammalian signal sequence of

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hCG- β and to insert a restriction site for Nde I. The sequence of the 5' primer was the following:
5'-AGGAGATATACATATGAGCAAGGAGCCGCTTCGGCCACGG-3'
(Sequence I.D. No. 1). The 3' antisense primer was
5 designed to incorporate a Sal I site into the hCG- β
gene and to add an extra stop codon to ensure
translation termination. The sequence of this primer
was: 5'-GCTGGGGTCGACTTATTATTGTGGGAGGATCGGGGT-3'
(Sequence I.D. No. 2). PCR reactions were performed
10 for 30 cycles where each cycle consisted of
denaturation at 95° for 1.5 min, primer extension at
53°C for 1.5 min and elongation at 75°C for 3 min.
Ten percent of a reaction was analyzed on a 1.2%
agarose gel to confirm that the size of the amplified
15 DNA was 471 base pairs. The remaining 90 μ l of the
reaction was concentrated to 10 μ l, resuspended in 200
 μ l of water, and concentrated again to 10 μ l in an
ultrafree-30 ultrafiltration filter (Millipore,
Bedford, MA) for the purpose of removing salts. The
20 concentrated and desalted DNA was diluted into a 40 μ l
reaction and digested with 16 units of Nde I and 20
units of Sal I restriction endonucleases for 2 hrs in
the following buffer: 50 mM Tris-HCl pH 8.0, 10 mM
MgCl₂, 50 mM NaCl. The digested DNA insert was
25 purified on a 0.7% low melting agarose gel, stained
with ethidium bromide, and excised from the gel for
use in the ligation reaction. To prepare the pET-12a
vector for ligation, 3 μ g of the plasmid was digested
with 8 units of Nde I in the Tris-HCl listed above for
30 2 hrs at 37°C. The NaCl concentration was raised to
150 mM and 10 units of Sal I were added for a second 2
hr digestion at 37°C. The linearized plasmid was
purified on a 0.7% low melting agarose gel, stained
with ethidium bromide and excised from the gel.
35 Approximately 5% of the gel slices containing the
linearized vector and the restriction enzyme-digested

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hCG- β gene were combined with 1 unit of T4 DNA ligase in a 20 μ l reaction with 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM DTT, and 0.5 mM ATP. The ligation reaction was performed overnight at room temperature and was then used to transfect HB101 *E. coli* cells made competent by the CaCl₂ method (Sambrook et al., 1989, *supra*). Colonies were isolated from LB agar plates containing 0.2 mg/ml ampicillin, and used to prepare 3 ml cultures in LB broth. Plasmid preps were prepared from these cultures (Sambrook et al., 1989, *supra*) and screened for the presence of the hCG- β gene by digestion with XbaI and Bam HI endonucleases. Plasmid from a positive clone was used to transfect BL21 (DE3) competent cells (Novagen, Madison, WI) according to the manufacturer's instructions.

**c. Expression and Purification of
Recombinant hCG- β**

BL21(DE3) colonies that contained the hCG- β /pET plasmid were grown up overnight in LB medium with 0.05 mg/ml ampicillin or carbenicillin (Sigma Chemical Co., St. Louis, MO). Cell suspensions were diluted 1:100 in LB medium containing antibiotic and grown to an OD₆₀₀ of 0.3-0.6 before induction with 0.4 mM IPTG. Cells were induced for 4 hrs at 37°C. Expression of hCG- β after induction was confirmed by Western Blot analysis of induced cells that had been fractionated on reducing SDS-PAGE gels. To prepare rehCG- β for refolding studies, cells from a 200 ml induced culture were pelleted and lysed in 10 ml in the presence of 1 mg/ml lysozyme, 30 mM Tris-HCl, pH 8.1, 1 mM EDTA, and 20% w/v sucrose for 10 min on ice. The protoplasts were then pelleted, resuspended by homogenization, and sonicated for 5 x 2 sec. This suspension was spun at 17,000 x g for 10 min at 4°C and then homogenized in 14 ml of 50 mM ammonium

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acetate, pH 5.0, 1 mM EDTA, and 1% Triton X-100. The homogenate was spun at 17,000 x g again for 10 min at 4°C, homogenized in 14 ml of 20 mM Tris-HCl, pH 8.1, 5 mM EDTA, and 2% deoxycholate, and rotated for 20 min at room temperature (Langley et al., Eur. J. Biochem., 163: 313-321, 1987). We have found that the deoxycholate selectively solubilizes lipids while leaving most of the hCG- β in the inclusion bodies. The inclusion bodies were recovered by centrifugation at 17,000 x g for 10 min at 22°C, washed with 20 mM Tris-Cl, pH 8.1, 5 mM EDTA, and recovered by centrifugation at 4°C. The purified inclusion bodies were solubilized in 1.5 ml 8 M urea, 50 mM DTT, 50 mM Tris-Cl, pH 8.7 at 37°C for 1 h under argon. This was cleared by centrifugation at 17,000 x g for 10 min at 22°C. The supernatant was injected onto a Vydac 300-Å, 5- μ m C₄ reversed-phase HPLC column (0.46 X 25 cm) equilibrated in 0.1% trifluoroacetic acid. The column was eluted isocratically for 10 min with 18% acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 1 ml/min followed by a 0.36%/min acetonitrile gradient for 90 min. Peak fractions were concentrated by speed-vac centrifugation, pooled, and the protein concentration determined by the method of Bradford. In this way, 1.2-1.4 mg of unfolded hCG- β was recovered from a 200 ml culture of cells. The protein was stored at -70°C until use in refolding assays.

d. rehCG- β Folding and Assembly with hCG- α

hCG- β was diluted to 1.28 μ M (0.02 mg/ml) in 50 mM Tris-Cl, pH 8.7, 1 mM EDTA, 0-2 M urea. Folding was initiated by the addition of 6.4 mM cysteamine and 3.6 mM cystamine that had been prepared as 10x concentrated stocks in 10 mM HCl immediately prior to use. Reactions were incubated for 1 min to 4 h at

room temperature under argon and folding was stopped by the addition of 100 mM sodium iodoacetate, 50 mM Tris-HCl, pH 8.7. Folded recombinant hCG- β (rehCG- β) was separated from unfolded rehCG- β on nonreducing SDS-PAGE gels as described previously (Ruddon et al., 1987, *supra*), and the bands were visualized by silver staining. We have found that the efficiency of folding varies depending on the length of time that the unfolded precursor is stored at -70°C. Optimum efficiencies were obtained when the protein was refolded within 1 day of HPLC purification. To form the rehCG- β /hCG- α dimer, urinary hCG- α was added to the refolding reactions at a final concentration of 1-1.4 μ M in the absence of urea. The extent of assembly was monitored by separating unassembled subunits from the dimer on nonreducing SDS-PAGE gels at 4°C (Beebe et al., *Endocrinology* 126: 384-391, 1990). In experiments to prepare rehCG- β / α for biological assays, folding and assembly reactions were not treated with iodoacetate.

e. Purification of rehCG- β / α Dimer

40 μ g of hCG- β and 40 μ g of a α were assembled in a 3.5 h reaction as described above. The 2 ml reaction was injected onto a Waters Protein Pak DEAE column (10 X 100 mm, 1000-Å pore size) equilibrated in 20 mM sodium phosphate buffer, pH 7.1. hCG dimer was eluted during a 0-1 M NaCl gradient in 20 mM phosphate buffer, pH 7.1 over 100 min. Elution positions of hCG and its unassembled subunits were first standardized using [³⁵S]cysteine-labelled hCG- β and hCG- α (data not shown). Next, elution of the unlabelled hCG formed using rehCG- β and urinary α was confirmed by SDS-PAGE by aliquots of the anion exchange fractions. Unassembled hCG- α eluted in the column void volume, whereas the hCG dimer eluted at 18

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min. Unassembled rehCG- β was not detected since most had either assembled with hCG- α or had formed large molecular weight aggregates (see Fig. 7, Lane 8) that did not elute from the anion exchange column in a sharp peak. The amount of hCG dimer recovered was estimated by separating the subunits of the dimer on a reducing gel and comparing the intensities of the silver-stained bands to that of both urinary hCG- α and rehCG- β standards that were analyzed on the same gel. Intensities were measured using the BioImage 1105 system with Whole Band Software (4.6 P, Millipore Corp., Bedford, MA) and were found to be linear in the range of protein concentrations examined. About 5 μ g of HPLC-purified hCG dimer was obtained from an assembly reaction that contained 40 μ g of both α and unfolded rehCG- β .

f. Receptor Binding and Activation Assays

The ability of rehCG- β / α dimer to bind to the CG/LH receptor and stimulate cAMP production in human fetal kidney "293" cells that had been transfected with the CG/LH receptor gene was assayed as described previously (Dunkel et al., 1993, *supra*).

g. In vivo Hormone Assays

Immature female Sprague-Dawley rats were hypophysectomized and implanted with a silastic capsule containing about 10 mg diethylstilbestrol at 22 days of age by a commercial breeder (Johnson Laboratories, Bridgeview, IL). The rats were shipped to our laboratory at 25 days of age and housed in standard vivarium facilities, with laboratory chow and 0.9% saline available *ad libitum*. Room temperature (24°C) and lighting (lights on, 0600-1800 h daily) were controlled throughout the studies.

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At 26 days of age, the rats received a priming dose of 10 IU pregnant mare serum gonadotropin (PMSG) (Calbiochem, La Jolla, CA), sc, at 0900 h. To assess *in vivo* bioactivities of recombinant hCG- β /urinary α , varying amounts of the recombinant hCG- β /urinary α or purified hCG (Lot No. CR-127) were injected subcutaneously fifty-two hours after PMSG priming. Control animals received no hormone after PMSG priming. At 18-20 h after treatment with hCG preparations, ovulatory potency of these hormones was checked by removing both oviducts to examine the number of ovulated oocytes.

h. Statistical Analysis

Each experiment was repeated at least two times and results are presented as the mean \pm SEM of more than three replicate determinations. Statistical analysis was done using Student's t test and $P < 0.05$ was considered as significantly different from corresponding controls.

II. RESULTS

a. Bacterial Expression and Purification of hCG- β

Restriction sites for the enzymes Nde I and Sal I were introduced into the hCG- β cDNA and the nucleotides that encode the signal sequence were removed by the polymerase chain reaction. The hCG- β gene was ligated into a pET expression vector that was used to transfect HB101 *E. coli* cells, used for large scale plasmid preparations. BL21(DE3) cells, the host for protein expression, were transfected with the pET-hCG- β plasmid according to the manufacturer's instructions. To express the hCG- β protein, the transfected BL21(DE3) cells were grown to log phase ($OD_{600} = 0.4-0.6$) and then induced with IPTG for 3-4 hrs

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at 37°C. Analysis of the detergent-solubilized cells by reducing SDS-PAGE showed the induction of a protein with a M_r of 15,000 (Fig. 3A), and Western Blot analysis confirmed that this protein was hCG- β (Fig. 3B). Cell fractionation studies confirmed that this protein was located in the inclusion bodies.

The purification of the hCG- β protein was based on first purifying the inclusion bodies. These were washed with Triton-X 100 and then with sodium deoxycholate to remove lipids, lipopolysaccharides, glycolipids and contaminating proteins. SDS-PAGE analysis of the washed inclusion bodies showed that hCG- β accounted for ~90% of the protein in the inclusion bodies (Fig. 4A, lane 1). hCG- β was solubilized by reduction in 50 mM DTT, 8 M urea, 50 mM Tris-HCl, pH 8.7 at 37°C under argon for 1 h. Removal of urea and contaminating proteins was achieved by reversed-phase HPLC resulting in a nearly homogeneous preparation of protein (Fig. 4, lane 2). The yield of hCG- β employing this expression and purification scheme was 1.3 mg protein from a 200 ml culture of bacteria, for a yield of 6-7 mg/l. This above procedure has been scaled up to 2.5 l of cultured cells, resulting in a ~4-fold greater yield of unfolded hCG- β (i.e., ~25 mg/l).

**b. Refolding of Recombinant hCG- β and
Assembly with Urinary hCG- α**

As described in Example 1, we have previously determined that the *in vitro* folding pathway of glycosylated hCG- β is the same as its intracellular folding pathway. Furthermore, we have identified optimal redox conditions for the disulfide bond formation that is required for folding of hCG- β (Example 1). In particular, a redox buffer composed of the oxidant cystamine and the reductant cysteamine

assembly of the hCG subunits. In the latter case, assembly seems to be facilitated by reduction of the carboxy-terminal disulfide bonds of hCG- β (Huth et al., 1993, *supra*). This cysteamine/cystamine redox buffer at alkaline pH was used to fold rehCG- β that had been purified by reversed-phase HPLC and concentrated by speed-vac centrifugation. Shown in Fig. 5 is a nonreducing SDS-PAGE analysis of folding intermediates that are involved in the hCG- β folding pathway. Within seconds of addition of the redox buffer, rehCG- β converts to a form that migrates faster on nonreducing SDS-PAGE gels than does the completely reduced and alkylated form of the protein (Fig. 5, lanes 2 and 3). This shift in migration is analogous to that observed for the conversion of reduced and denatured, glycosylated hCG- β to the first folding intermediate, p β 1-early (Huth et al., 1992a, *supra*). As folding of rehCG- β continued, the form at the p β 1 locus converted to a lower migrating form (Fig. 5, lanes 4-7) that is analogous to the p β 2 intermediates (indicated as "folded β " in Fig. 5) found in the folding pathway of the glycosylated protein (Ruddon et al., 1987, *supra*). Dimers, trimers, tetramers, and larger molecular weight aggregates of rehCG- β also formed during the folding reaction (Fig. 5, lanes 4-7) and are probably a result of intermolecular disulfide bond formation since they were not observed on reducing gels. Interestingly, the amount of aggregate formation was reduced when rehCG- β was folded in the presence of 2 M urea (Fig. 5, lanes 8-12). The percent folding under these reaction conditions was estimated in two ways: 1) by comparing the intensity of the folded p β 2 bands to the intensity of the unfolded precursor (lane 2) and 2) by comparing the intensity of the p β 2 bands (lanes 4-7)

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to the total intensity of stained proteins in the same lanes. In this way, the folding efficiency was found to be 40-60% in the presence of 2M urea, which is comparable to the efficiency achieved for refolding the glycosylated, reduced and denatured form of hCG- β (Huth et al., 1993, *supra*).

Folding plus assembly of rehCG- β was carried out in the cysteamine/cystamine redox buffer in the presence of urinary hCG- α . A band was observed on nonreducing SDS-PAGE gels, run at 4°C (Beebe et al., 1990, *supra*), that migrated at the appropriate molecular weight of a urinary hCG- α /nonglycosylated hCG- β dimer (Fig. 6, lanes 5-8). Interestingly, assembly was almost completely inhibited in the presence of 2M urea (Fig. 6, lanes 9-13), whereas the efficiency of the folding reaction was increased under these conditions (Fig. 7). To confirm that the putative dimer was composed of both α and β hCG subunits and to obtain the recombinant hCG dimer for biological assays, this protein was purified by anion exchange HPLC to >90% purity (Fig. 7). This preparation was then used to confirm the presence of both hCG- α and - β in the isolated protein. The subunits were separated on a reducing SDS-PAGE gel, blotted to PVDF membranes, and probed with an antibody to either the hCG- α or hCG- β subunit. The results of these Western blots confirmed that both the α and β subunit were present in the hCG dimer prior to subunit dissociation (Fig. 8).

c. Biological Activity of hCG- α /rehCG- β

The biological activity of this form of hCG was evaluated in receptor binding and cAMP stimulation assays. As shown in Fig. 9A, rehCG competed for the binding of urinary hCG to its receptor. Furthermore,

rehCG maximally stimulated the production of cAMP in human fetal kidney cells that had been transfected with the hLHR, and was approximately equipotent to wild-type hCG (Fig. 9B).

5 To assess the *in vivo* bioactivity of recombinant hCG- β /urinary α , we injected varying amounts of the recombinant hCG- β /urinary α sc to immature hypophysectomized female rats primed with PMSG 52 h earlier. Purified hCG (CR-127) was used as
10 a positive control, and saline was used as the negative control. As shown in Fig. 10, subcutaneous injection of saline did not cause any animals to ovulate. However, treatment with earlier purified hCG or rehCG resulted in dose-dependent increases in the
15 number of oocytes per ovulating animal. Although the present data does not demonstrate parallel dose-response curves between animals treated with purified hCG and recombinant hCG- β /urinary α , we estimated the apparent ED_{50} to be 0.3 μ g for purified hCG and 1 μ g
20 for rehCG, suggesting that rehCG is approximately 3-fold less potent in ovulation induction than hCG.

 These *in vitro* and *in vivo* results indicate that rehCG- β folded and assembled with hCG- α in a conformation very similar to that of glycosylated hCG-
25 β that is made in human cells.

 The present invention is not limited to the particular embodiments described and exemplified, but is capable of variation and modification within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: University of Nebraska
Ruddon Jr., Raymond W. (U.S. only)
Huth, Jeffrey R. (U.S. only)
- (ii) TITLE OF INVENTION:
Biologically Active Glycoprotein Hormones
Produced in Procaryotic Cells
- 10 (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
15 (A) ADDRESSEE: Dann, Dorfman, Herrell &
Skillman
(B) STREET: 1601 Market Street, Suite 720
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19103-2307
- 20 (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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- (viii) ATTORNEY/AGENT INFORMATION:
35 (A) NAME: Reed, Janet E.
(B) REGISTRATION NUMBER: 36,252
(C) REFERENCE/DOCKET NUMBER: UNMC 63065
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (215) 563-4100
(B) TELEFAX: (215) 563-4044

(2) INFORMATION FOR SEQ ID NO:1:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 40 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGAGATATA CATATGAGCA AGGAGCCGCT TCGGCCACGG
 40

(2) INFORMATION FOR SEQ ID NO:2:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGGGGTCG ACTTATTATT GTGGGAGGAT CGGGGT
 36

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What is claimed is:

1. A glycoprotein hormone subunit produced in procaryotic cells, said subunit being capable of assembling with a counterpart subunit to form a glycoprotein hormone having at least one activity of a naturally occurring glycoprotein hormone.

2. A glycoprotein hormone subunit as claimed in claim 1, selected from the group of glycoproteins consisting of chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone.

3. A glycoprotein hormone subunit as claimed in claim 2, which is a subunit of human chorionic gonadotropin.

4. A glycoprotein hormone subunit as claimed in claim 1, which is an α subunit.

5. A glycoprotein hormone subunit as claimed in claim 1, which is a β subunit.

6. A glycoprotein hormone subunit as claimed in claim 1, wherein said at least one activity is a receptor binding activity.

7. A glycoprotein hormone subunit as claimed in claim 1, wherein said at least one activity is a signal transducing activity.

8. A recombinant glycoprotein hormone comprising an α subunit and a β subunit, at least one of said subunits being produced in procaryotic cells, said recombinant glycoprotein hormone having at least

10 claim 8, wherein said α subunit is produced in
procaryotic cells.

12. A glycoprotein hormone as claimed in
claim 8, wherein said β subunit is produced in
procaryotic cells.

15 13. A glycoprotein hormone as claimed in
claim 8, wherein both said α and said β subunits are
produced in procaryotic cells.

14. A glycoprotein hormone as claimed in
claim 8, selected from the group consisting of
20 chorionic gonadotropin, luteinizing hormone, follicle
stimulating hormone and thyroid stimulating hormone.

15. A glycoprotein hormone as claimed in
claim 14, wherein said α subunit and said β subunit
are selected from the same member of the group
25 consisting of chorionic gonadotropin, luteinizing
hormone, follicle stimulating hormone and thyroid
stimulating hormone.

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16. A glycoprotein hormone as claimed in claim 15, which is human chorionic gonadotropin.

5 17. A glycoprotein hormone as claimed in claim 14, wherein said α subunit and said β subunit are selected from different members of the group consisting of chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone.

10 18. A glycoprotein hormone as claimed in claim 17, wherein said α subunit is human chorionic gonadotropin α subunit and said β subunit is a subunit other than human chorionic gonadotropin β subunit.

15 19. A glycoprotein hormone as claimed in claim 17, wherein said β subunit is human chorionic gonadotropin β subunit and said α subunit is a subunit other than human chorionic gonadotropin α subunit.

20. A glycoprotein hormone β subunit, which is fully deglycosylated.

20 21. A glycoprotein hormone subunit as claimed in claim 20, selected from the group consisting of chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone.

25 22. A glycoprotein hormone subunit as claimed in claim 21, which is chorionic gonadotropin β subunit.

23. A fully deglycosylated glycoprotein hormone.

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24. A fully deglycosylated glycoprotein hormone as claimed in claim 23, selected from the group consisting of chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone.

25. A fully deglycosylated glycoprotein hormone, which is chorionic gonadotropin.

26. A glycoprotein hormone having a fully deglycosylated subunit and a fully glycosylated subunit.

27. A pharmaceutical preparation comprising a glycoprotein hormone as claimed in claims 8, 23 and 26, in a biologically compatible medium.

28. A method of making a glycoprotein hormone subunit capable of assembling with a counterpart subunit to form a glycoprotein hormone, said method comprising the steps of:

- a) transforming a procaryotic cell with a nucleic acid molecule comprising a coding sequence for said subunit, operably linked to regulatory sequences for expressing said coding sequence in said procaryotic cell;
- b) expressing said coding sequence in said procaryotic cell to produce an unfolded form of said subunit;
- c) purifying said subunit from other components of said procaryotic cell; and
- d) folding said subunit by dispersing said subunit in a thiol redox buffer having a redox potential of between about -145 and about -200 mV, to enable ordered formation of disulfide bonds, thereby forming an assembly-competent glycoprotein hormone

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subunit capable of assembling with a counterpart subunit to form a glycoprotein hormone.

29. A method according to claim 28, wherein said procaryotic cell is *E. coli*, and said nucleic acid molecule is disposed within a pET expression vector.

30. A method according to claim 29, wherein said subunit is produced in inclusion bodies of said *E. coli* cell.

31. A method according to claim 30, wherein said purification of said subunit comprises the steps of:

- i) purifying said inclusion bodies from said other components of said procaryotic cell;
- ii) solubilizing said inclusion bodies to form a soluble fraction comprising said subunit, and an insoluble fraction;
- iii) separating said soluble fraction from said insoluble fraction; and
- iv) isolating said subunit from said soluble fraction.

32. A method according to claim 31, wherein the step of purifying said inclusion bodies comprises:

- 1) lysing said *E. coli* cells to produce protoplasts comprising said inclusion bodies;
- 2) homogenizing said protoplasts to produce a protoplast homogenate;
- 3) exposing said protoplast homogenate to deoxycholate in an amount effective to selectively solubilize lipids in said protoplast homogenate while leaving substantially all of said subunit in said inclusion bodies; and

4) separating said inclusion bodies from other components of said protoplast homogenat , thereby purifying said inclusion bodies.

5 33. A method according to claim 28, wherein said thiol redox buffer has a pH of between about 7.4 and about 8.7.

34. A method according to claim 33, wherein said thiol redox buffer comprises a cysteamine/cystamine redox pair at a ratio of
10 cysteamine to cystamine of about 2 to 1.

35. A method according to claim 34, wherein said redox buffer comprises a combined concentration of cysteamine and cystamine of between about 2 mM and about 10 mM.

15 36. A method according to claim 34, wherein said thiol redox buffer has a pH of about 8.7 and comprises about 6.4 mM cysteamine and about 3.6 mM cystamine.

20 37. A method according to claim 33, wherein said thiol redox buffer comprises a reduced glutathione/oxidized glutathione redox pair, at a ratio of reduced glutathione to oxidized glutathione of about 6 to 1.

25 38. A method according to claim 37, wherein said thiol redox buffer comprises a total glutathione concentration of about 2 mM.

39. A method according to claim 37, wherein said thiol redox buffer further comprises protein disulfide isomerase.

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40. A method according to claim 37, wherein said thiol redox buffer has a pH of about 7.4 and comprises about 1.7 mM reduced glutathione, about 0.27 mM oxidized glutathione and about 1 mg/ml protein disulfide isomerase.

41. A method of making a glycoprotein hormone, said method comprising the steps of:

a) transforming a procaryotic cell with a nucleic acid molecule comprising a coding sequence for a subunit of said glycoprotein hormone, operably linked to regulatory sequences for expressing said coding sequence in said procaryotic cell;

b) expressing said coding sequence in said procaryotic cell to produce an unfolded form of said subunit;

c) purifying said subunit from other components of said procaryotic cell;

d) folding said subunit by dispersing said subunit in a thiol redox buffer having a redox potential of between about -145 and about -200 mV, to enable ordered formation of disulfide bonds, thereby forming an assembly-competent glycoprotein hormone subunit capable of assembling with a counterpart subunit to form a glycoprotein hormone;

e) contacting said assembly-competent glycoprotein hormone subunit with said counterpart subunit in said thiol redox buffer, said contacting causing assembly of said subunit with said counterpart subunit, thereby producing said glycoprotein hormone.

42. A glycoprotein hormone subunit capable of assembling with a counterpart subunit to form a glycoprotein hormone, produced by a method comprising:

a) transforming a procaryotic cell with a nucleic acid molecule comprising a coding

sequence for said subunit, operably linked to regulatory sequences for expressing said coding sequence in said procaryotic cell;

5 b) expressing said coding sequence in said procaryotic cell to produce an unfolded form of said subunit;

 c) purifying said subunit from other components of said procaryotic cell; and

10 d) folding said subunit by dispersing said subunit in a thiol redox buffer having a redox potential of between about -145 and about -200 mV, to enable ordered formation of disulfide bonds.

43. A recombinant glycoprotein hormone produced by a method comprising:

15 a) transforming a procaryotic cell with a nucleic acid molecule comprising a coding sequence for a subunit of said glycoprotein hormone, operably linked to regulatory sequences for expressing said coding sequence in said procaryotic cell;

20 b) expressing said coding sequence in said procaryotic cell to produce an unfolded form of said subunit;

 c) purifying said subunit from other components of said procaryotic cell;

25 d) folding said subunit by dispersing said subunit in a thiol redox buffer having a redox potential of between about -145 and about -200 mV, to enable ordered formation of disulfide bonds, thereby forming an assembly-competent glycoprotein hormone subunit capable of assembling with a counterpart subunit to form a glycoprotein hormone;

30 e) contacting said assembly-competent glycoprotein hormone subunit with said counterpart subunit in said thiol redox buffer, said contacting

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causing assembly of said subunit with said counterpart subunit.

5 44. A solution for folding an unfolded protein having at least one disulfide bond when folded, which comprises between about 1.3 and about 6.4 mM cysteamine and between about 0.7 and about 3.6 mM cystamine, having a ratio of cysteamine to cystamine of about 2 to 1, and having a pH of about 8.7.

10 45. A solution as claimed in claim 44, wherein said unfolded protein is a glycoprotein hormone subunit.

15 46. A method of folding an unfolded glycoprotein hormone subunit to form an assembly-competent subunit capable of assembling with a counterpart glycoprotein hormone subunit to produce a glycoprotein hormone, said method comprising dispersing said subunit in a solution comprising between about 1.3 and about 6.4 mM cysteamine and
20 between about 0.7 and about 3.6 mM cystamine, having a ratio of cysteamine to cystamine of about 2 to 1, and having a pH of about 8.7, to enable folding of said subunit by ordered formation of disulfide bonds, thereby forming said assembly-competent subunit.

25 47. A method of treating a patient having a condition that is relieved by increasing an amount of a glycoprotein hormone in said patient's body, said method comprising administering to said patient the pharmaceutical preparation of claim 27, in an amount
30 effective to relieve said condition.

...ing to claim 47, wherein
said condition is selected from the group consisting
of reduced fertility and hypogonadism, and said
glycoprotein hormone is a gonadotropin.

5 49. A method according to claim 47, wherein
said condition is hypothyroidism and said glycoprotein
hormone is thyroid stimulating hormone.

10 50. A method treating a patient having a
condition that is relieved by decreasing an amount of
a glycoprotein hormone or preventing binding of a
glycoprotein hormone to cellular receptors in said
patient's body, said method comprising administering
to said patient the pharmaceutical preparation of
claim 27, which comprises a deglycosylated form of
15 said glycoprotein hormone, in an amount effective to
relieve said condition.

20 51. A method according to claim 50, wherein
said condition is selected from the group consisting
of fertility and over-production of steroid hormones,
and said deglycosylated glycoprotein hormone is a
gonadotropin.

25 52. A method according to claim 50, wherein
said condition is hyperthyroidism and said
deglycosylated glycoprotein hormone is thyroid
stimulating hormone.

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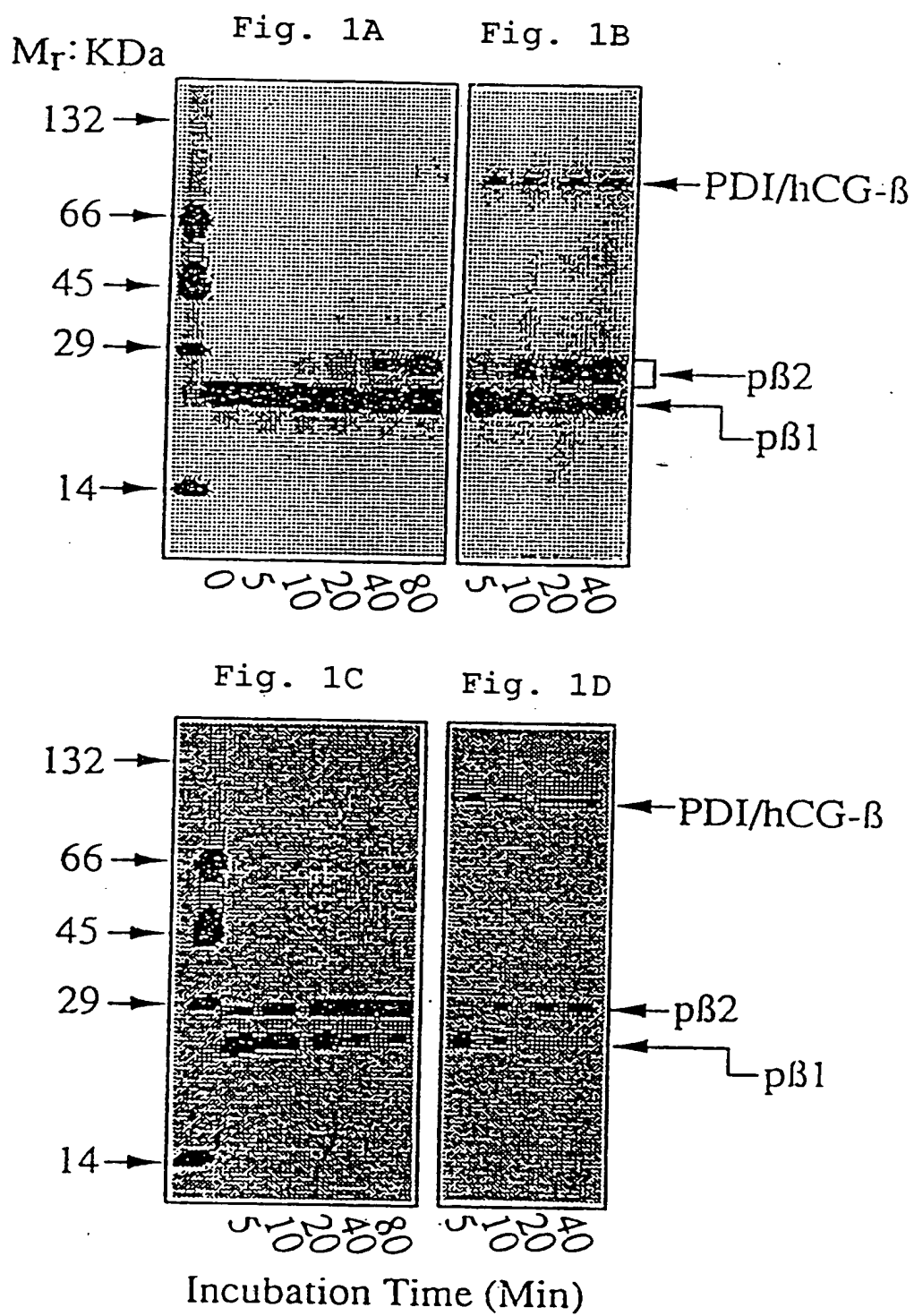


Figure 1

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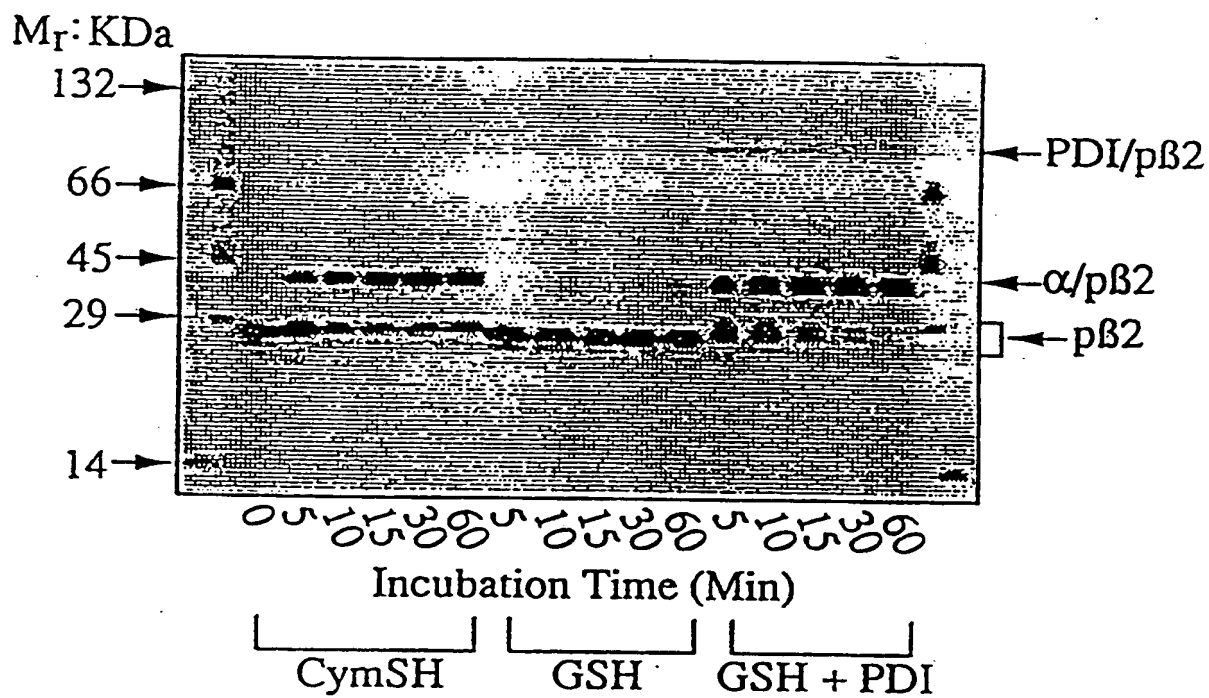


Figure 2

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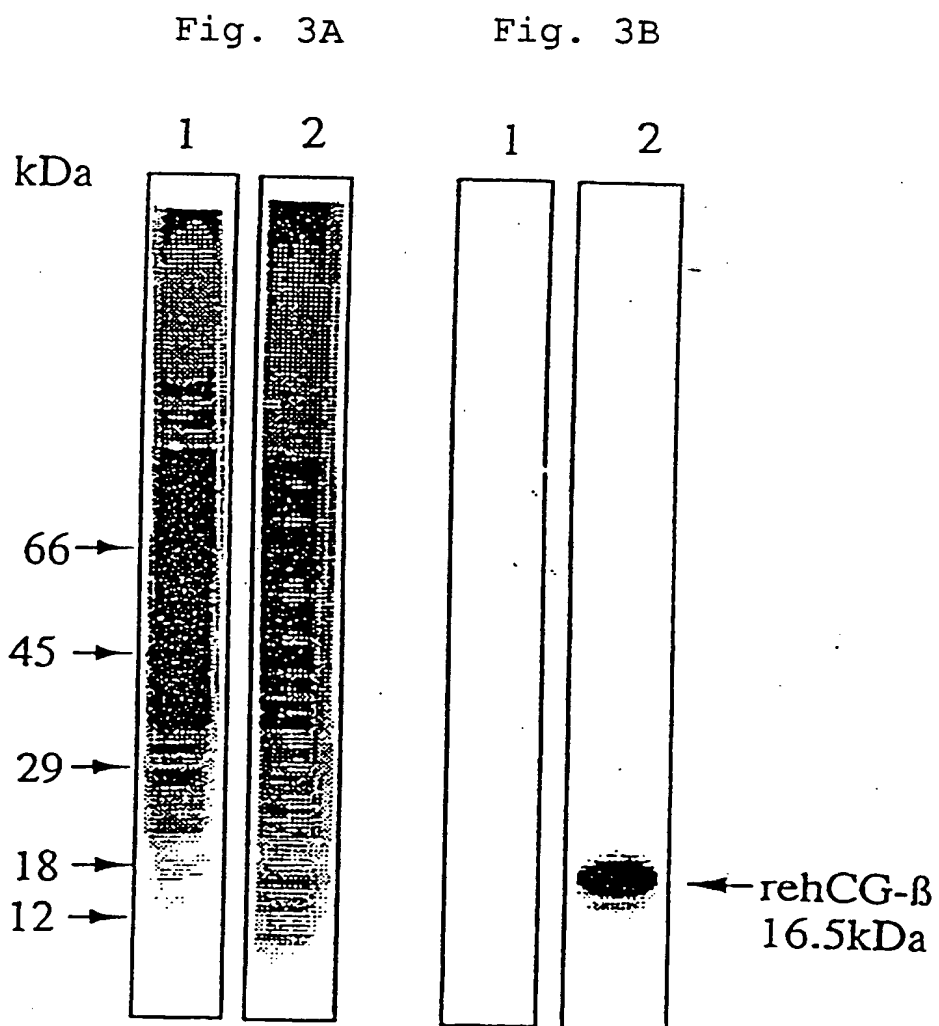


Figure 3

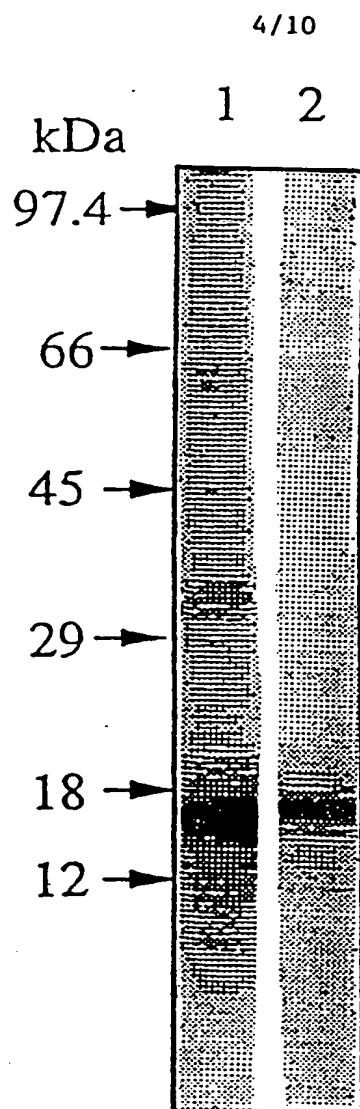


Figure 4

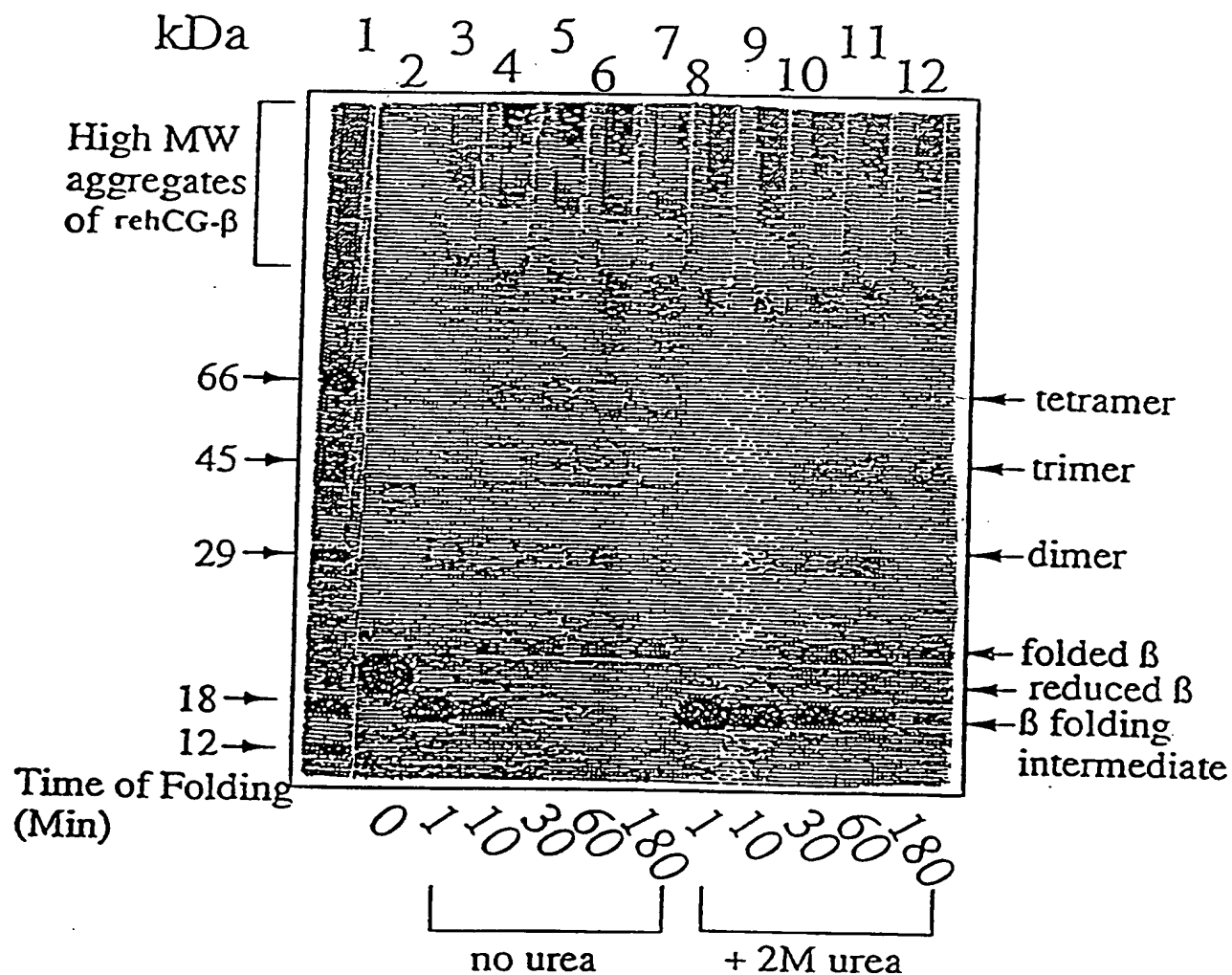


Figure 5

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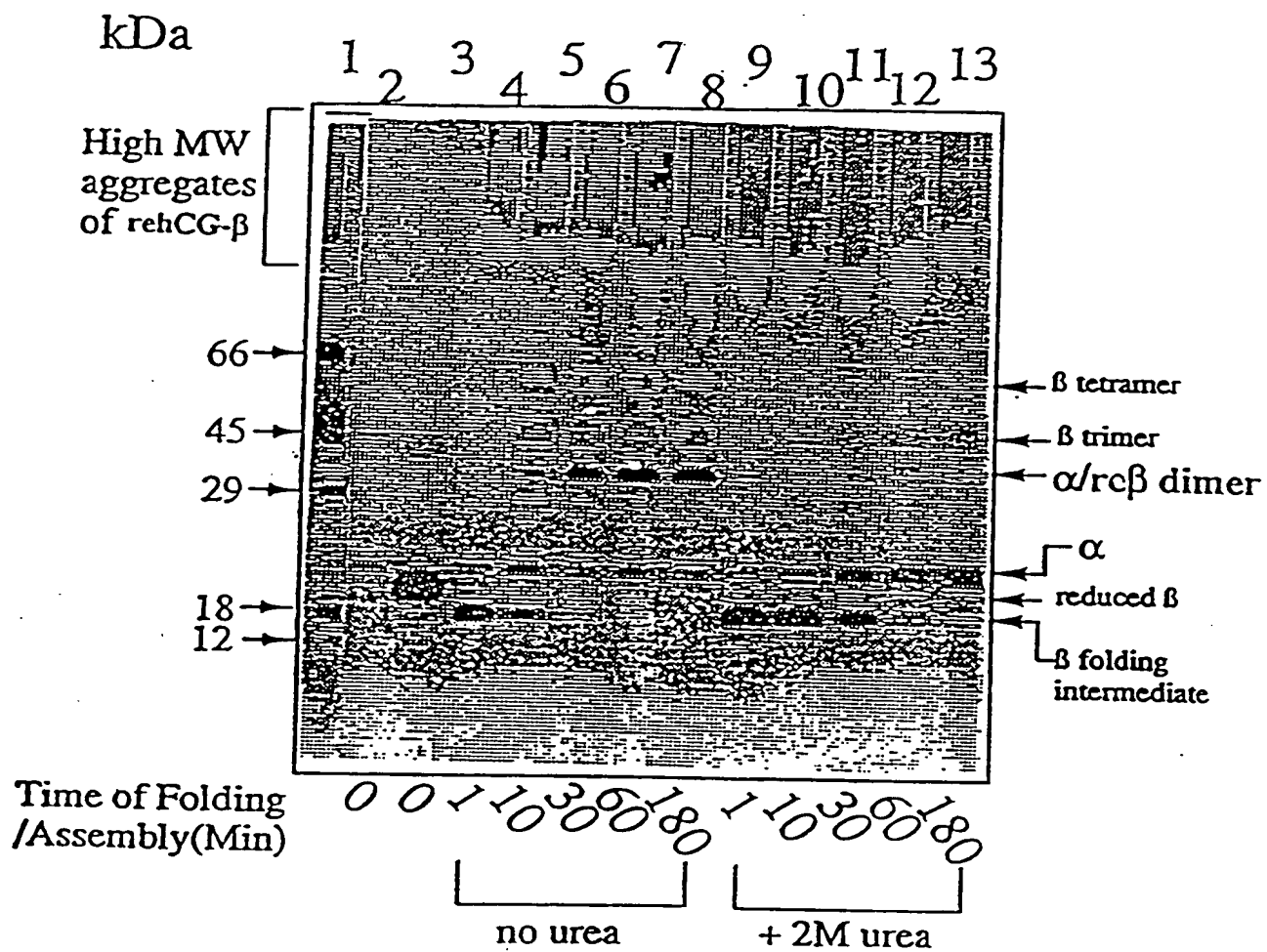


Figure 6

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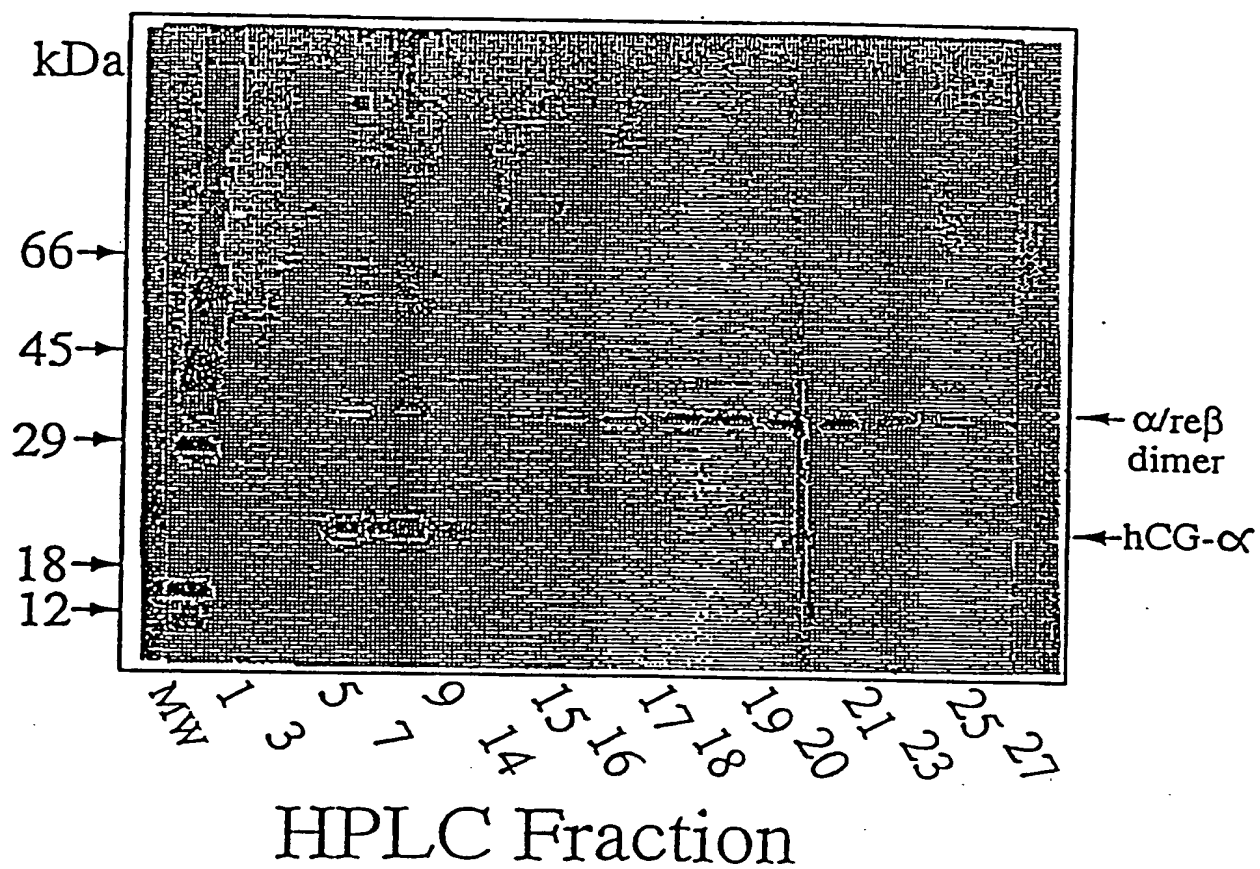


Figure 7

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Fig. 8A
Anti-hCG- α

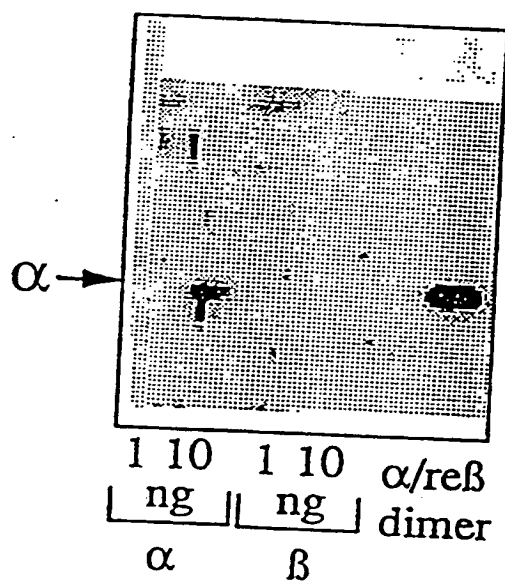


Fig. 8B
Anti-hCG- β

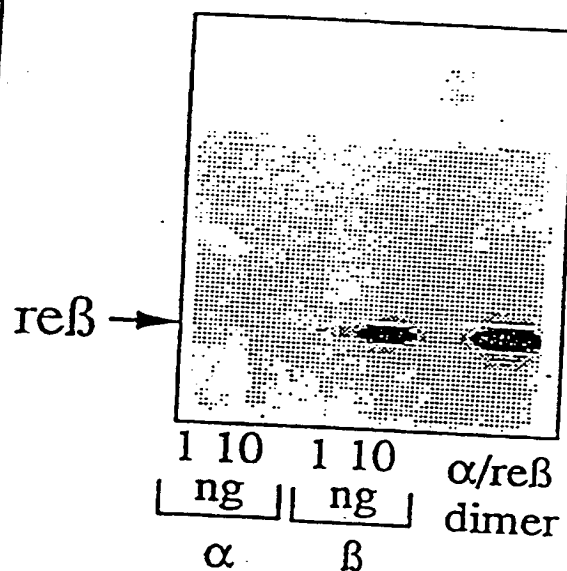


Figure 8

SUBSTITUTE SHEET (RULE 26)

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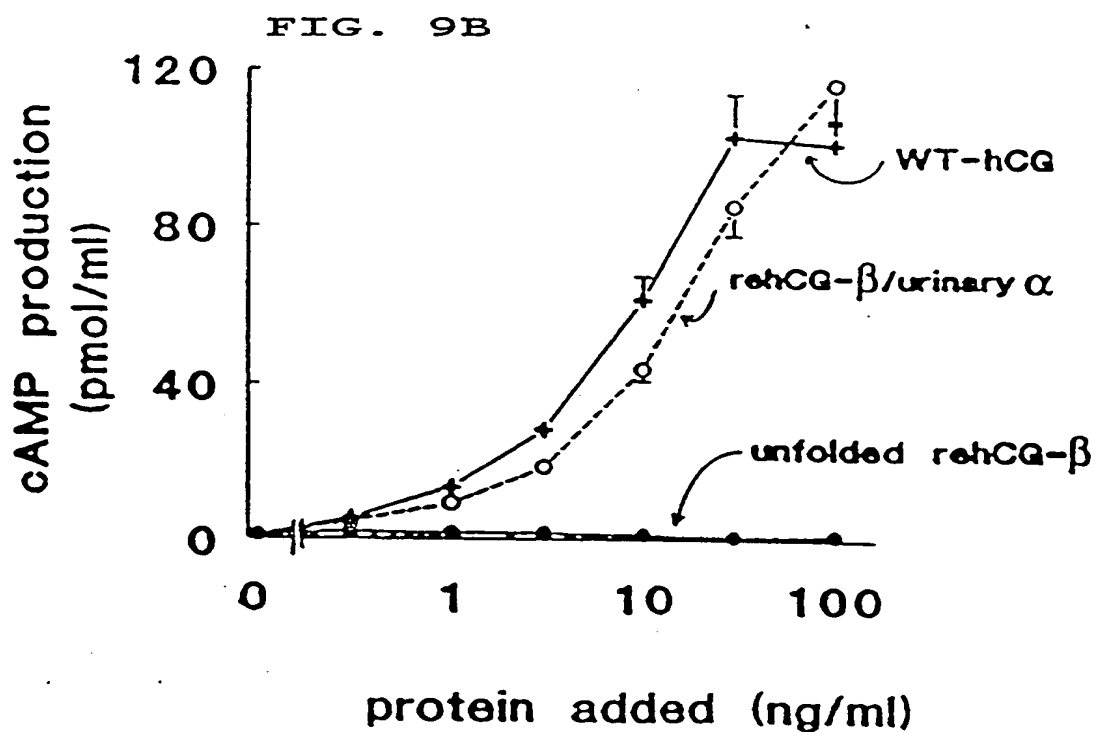
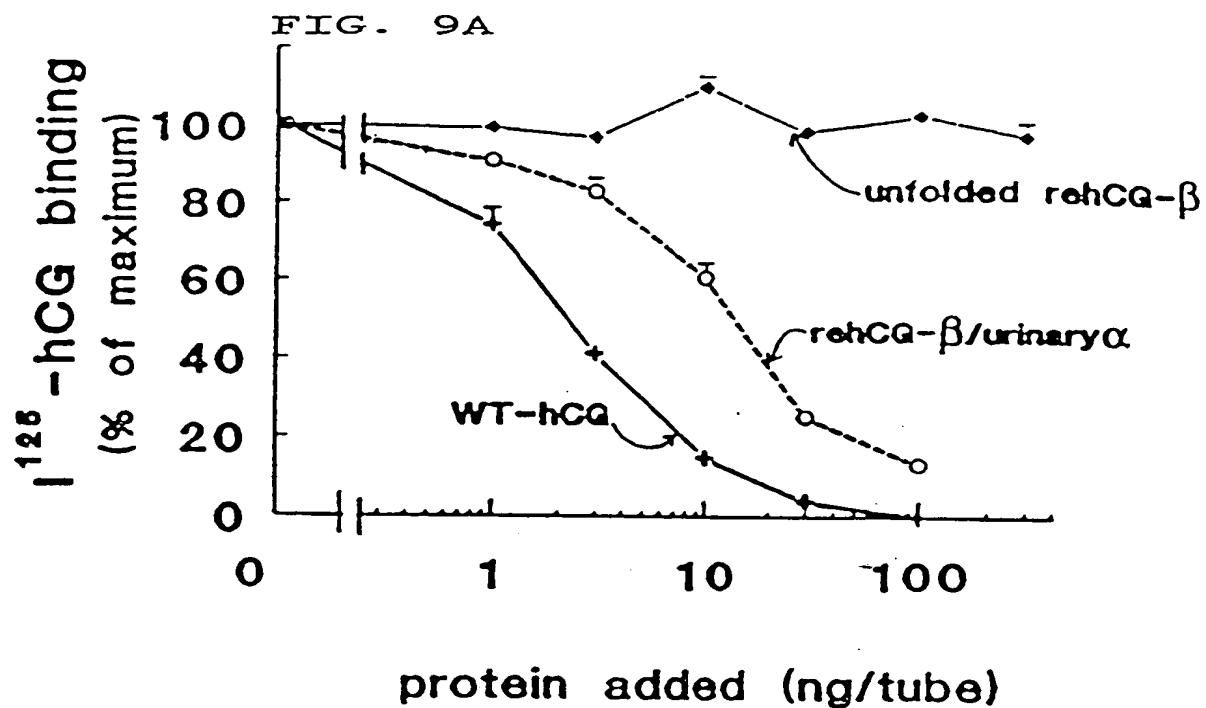


FIGURE 9

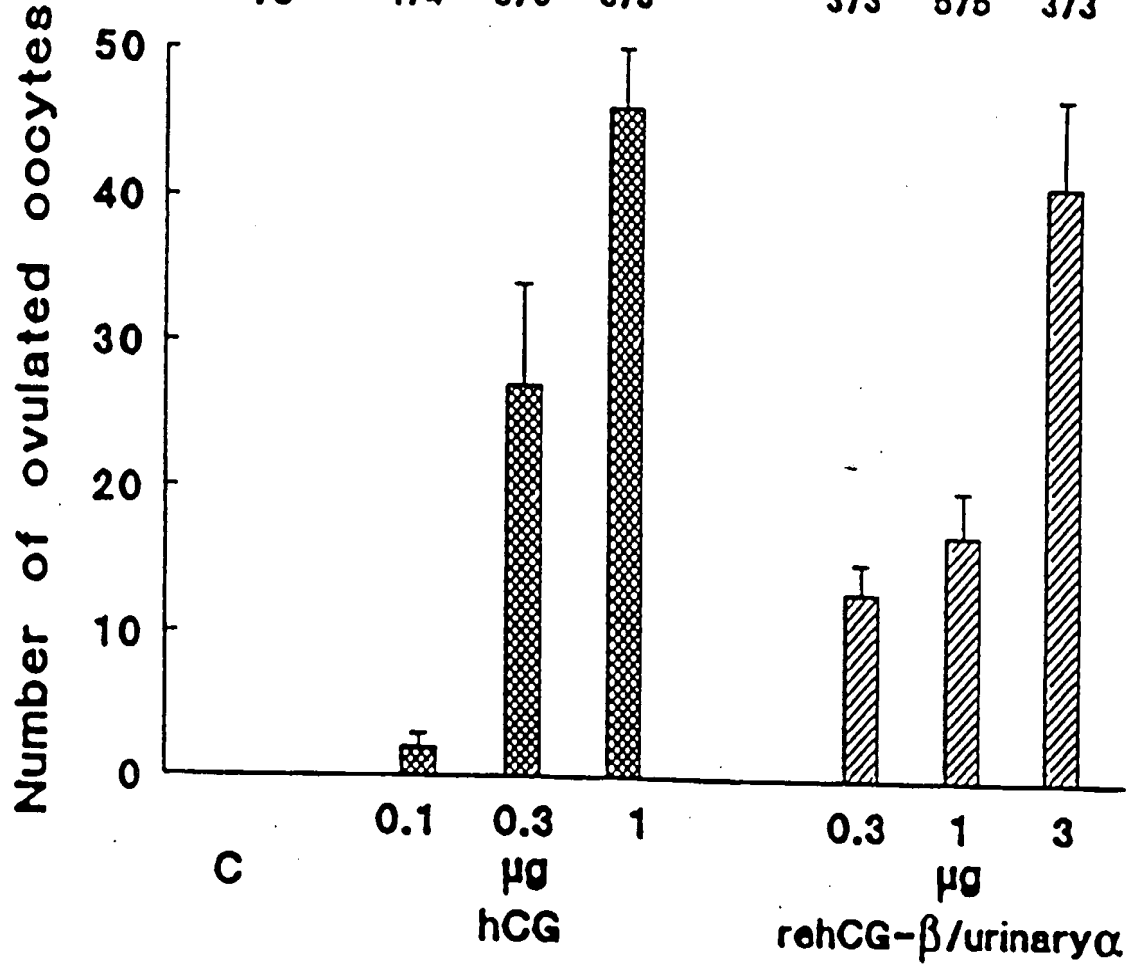


FIGURE 10

INTERNATIONAL SEARCH REPORT

Int. national application No.
PCT/US95/06616

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological chemistry, Volume 268, Number 22, issued 05 August 1993, J.R. Huth et al., "Protein folding and assembly <i>in Vitro</i> parallel intracellular folding and assembly", pages 16472-16482, entire document.	1-43
X	Endocrinology, Volume 135, No. 3, issued 1994, "Bacterial Expression and <i>in Vitro</i> folding of the β -subunit of human chorionic gonadotropin (hCG β) and functional assembly of recombinant hCG β with hCG α ", pages 911-918, entire document.	1-46

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search
17 AUGUST 1995

Date of mailing of the international search report
31 AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

LORRAINE M. SPECTOR

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06616

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Endocrinology, Volume 132, Number 2, issued 1993, L. Dunkel et al., "Deglycosylated human chorionic gonadotropin (hCG) antagonizes hCG stimulation of 3',5'-cyclic adenosine monophosphate accumulation through a noncompetitive interaction with recombinant human luteinizing hormone receptors", pages 763-769, entire document.	8, 9, 11-17, 19-25, 27, 42, 43
X --- Y	J. Cellular Biochemistry, Volume 29, issued 1985, T.W. Strickland et al., "The common α subunit of bovine glycoprotein hormones: limited formation of native structure by the totally nonglycosylated polypeptide chain", pages 55-67, entire document, especially sentence bridging pages 56-57, pages 61-62.	1-4, 6-11, 14-17, 19, 26, 27 ----- 5, 12, 13, 18, 20-25, 28-46
X --- Y	US, A, 4,977,248 (CREIGHTON) 11 December 1990, entire document, especially column 5.	44, 45 ----- 1-43, 46
X --- Y	US, A, 4,599,197 (WETZEL) 08 July 1986, entire document, especially column 16, lines 38-40.	44, 45 ----- 1-43, 46
X --- Y	US, A, 4,766,205 (GHOSH-DASTIDAR) 23 August 1988, entire document, especially column 7, lines 10-57.	44, 45 ----- 1-43, 46
X --- Y	US, A, 4,620,948 (BUILDER ET AL.) 04 November 1986, entire document, especially columns 16 and 17.	44, 45 ----- 1-43, 46
Y	WO, A, 90/09800 (BOIME ET AL.) 07 September 1990, entire document.	1-46
Y	EP, A, 0 322 226 (CHAPPEL ET AL.) 28 June 1989, see abstract.	8-19, 26, 27-41, 44-46
X	Molecular Cellular Endocrinology, Volume 79, issued 1991, V. Padmanabhan et al., "Follicle-stimulating hormone signal transduction: role of carbohydrate in aromatase induction in immature rat Sertoli cells", pages 119-128, entire document.	1, 2, 4-15, 17, 18, 20, 21, 23, 24, 26, 27, 42, 43

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06616

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-46
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

IPC (6):

C07K 1/00, 14/59; C12P 21/00; C12N 15/16, 1/21; C07C 291/00; A61K 39/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/397; 435/69.4, 172.1, 172.3, 252.3, 252.33; 546/7; 568/21, 61; 414/198.1

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

530/397, 402, 405, 412, 419; 435/69.4, 172.1, 172.3, 240.2 252.3, 252.33; 546/7; 568/21, 61; 414/198.1

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE

search terms: glycoprotein hormones, LH, FSH, TSH, CG, glycosylation, recombinant production.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-27, 42, 43, and 50-52 drawn to non-glycosylated glycoprotein hormones or subunits thereof, or dimers comprising such subunits.

Group II, claims 28-41, drawn to recombinant production of non-glycosylated glycoprotein subunits in prokaryotic cells.

Group III, claims 44, 45, drawn to cysteamine solutions for protein folding.

Group IV, claim 46, drawn to a method for folding a glycoprotein hormone.

Group V, claims 47-49, drawn to In vivo methods of treatment using non- or partially-glycosylated glycoprotein hormones.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventive concept of group I, non-glycosylated glycoprotein hormones is not linked to the inventive concept of group II, production of such using recombinant DNA technology and using prokaryotic cells as hosts, because the products of group I may be made by distinct means such as enzymatic deglycosylation. Descriptive language is used in the product claims to create the perception of a shared critical feature that does not exist. The solution of group III can be used with various proteins, and have no unifying technical feature with any of the other inventions. The method of group IV does not share a unifying technical feature with the other inventions as it is not limited to the folding of non-glycosylated glycoprotein hormones. The methods of group V do not share a unifying technical feature with the products of group I because lack of glycosylation is not considered to be the inventive concept because fully glycosylated forms of the hormones would have similar effect. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.